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- (54) **PHOSPHOLIPASE A₂ INHIBITING PROTEIN ORIGINATING IN INFLAMED REGION, PRODUCTION THEREOF, AND GENE THEREFOR.**
- (57) A phospholipase A₂ inhibiting protein originating in inflamed regions and having a sequence of 334 amino acids as shown or a physiologically equivalent amino acid sequence, a method of producing said protein by enzymatically treating the serum of a mammal, and a gene which codes for said protein.

FIG 1-1

Glu Asp Val Pro Ala Ala Asp Leu Ser Asp Glu Val 12
 Pro Asp Thr Asp Ser Glu Thr Arg Ile Leu Leu Glu
 Gly Thr Pro Val Ala Glu Met Ala Glu Asp Ala Val
 Asp Gly Glu Arg Leu Lys His Leu Ile Val Thr Pro
 Ser Gly Cys Gly Glu Glu Asn Met Ile Gly Met Thr 60
 Pro Thr Val Ile Ala Val His Tyr Leu Asp Glu Thr
 Glu Glu Trp Glu Lys Phe Gly Leu Glu Lys Arg Glu
 Glu Ala Leu Glu Leu Ile Lys Lys Gly Tyr Thr Glu
 Glu Leu Ala Phe Lys Glu Pro Ser Ser Ala Tyr Ala 108
 Ala Phe Asn Asn Arg Pro Pro Ser Thr Trp Leu Thr
 Ala Tyr Val Val Lys Val Phe Ser Leu Ala Ala Asn
 Leu Ile Ala Ile Asp Ser Glu Val Leu Cys Gly Ala
 Val Lys Trp Leu Ile Leu Glu Lys Glu Lys Pro Asp 156
 Gly Val Phe Glu Glu Asp Gly Pro Val Ile His Glu

FIG 1-2

Glu Met Ile Gly Gly Phe Arg Asn Thr Lys Glu Ala 180
 Asp Val Ser Leu Thr Ala Phe Val Leu Ile Ala Leu
 Glu Glu Ala Arg Asp Ile Cys Glu Gly Glu Val Asn
 Ser Leu Pro Gly Ser Ile Asn Lys Ala Gly Glu Tyr
 Leu Glu Ala Ser Tyr Leu Asn Leu Glu Arg Pro Tyr 228
 Thr Val Ala Ile Ala Gly Tyr Ala Leu Ala Leu Met
 Asn Lys Leu Glu Glu Pro Tyr Leu Thr Lys Phe Leu
 Asn Thr Ala Lys Asp Arg Asn Arg Trp Glu Glu Pro
 Gly Glu Glu Leu Tyr Asn Val Glu Ala Thr Ser Tyr 276
 Ala Leu Leu Ala Leu Leu Leu Leu Lys Asp Phe Asp
 Ser Val Pro Pro Val Val Arg Trp Leu Asn Glu Glu
 Arg Tyr Tyr Gly Gly Gly Tyr Gly Ser Thr Glu Ala
 Thr Phe Met Val Phe Glu Ala Leu Ala Glu Tyr Glu 324
 Thr Asp Val Pro Asp His Lys Asp Leu Asn Met Asp

FIG 1-3

Val Ser Leu His Leu Pro Ser Arg 344

TECHNICAL FIELD

The invention relates to protein inhibiting phospholipase A₂ which participates in the progress of inflammation, the production process and gene therefor. More particularly, the invention concerns rat or human protein inhibitors of phospholipase A₂ purified from inflammatory sites.

5 Background Art

Steroids have been used in treatment for a variety of inflammatory diseases as a powerful anti-inflammatory agent. Steroids have, however, various kinds of side-effects because of its hormonal actions and its application must be limited to relatively serious cases. Thus, the development of a novel drug having
10 anti-inflammatory action comparative to that of steroids but much reduced side-effects has been most seriously awaited by the doctors treating inflammatory diseases.

As one of the mechanisms for steroids to manifest its anti-inflammatory action, it has been proposed that the synthesis of a protein which inhibits phospholipase A₂ is induced in the inflammatory site to suppress the production of arachidonic acid caused by the enzyme, resultantly the formation of the
15 arachidonic acid metabolites having pro-inflammatory activity is lowered [Flower et al. Nature 278, 456, (1979)]. The phospholipase A₂-inhibitory protein has been expected to have the activity satisfactory for the above-stated requirements and numerous researchers have tried the isolation and purification of the protein, and the evaluation on its inflammatory action.

Until now, however, no protein having powerful actions comparative to steroids has been found and the
20 following two reasons have been cited as the causes:

- (1) In the previous researches, the enzyme purified from pancreas has been utilized in the evaluation system for activity inhibition, but the protein inhibitors of phospholipase A₂ purified from inflammatory sites has a different structure and different activities from phospholipase A₂ originating from pancreas, and
- 25 (2) The inhibitor does not directly act on phospholipase A₂, and an apparent inhibitory activity by its interaction with the substrate has been detected.

The inventors paid attention to these problems and purified the phospholipase A₂ from the peritoneal exudate of rats with peritonitis caused by casein.

Additionally, the inventors have succeeded in purification of phospholipase A₂ from human inflammation
30 sites, namely the synovial fluid in humans with rheumatoid arthritis [W089/05851 specification (Japanese Patent 325255/ (1987), Hara et al, J. Biochem. 104, 326-328 1988), and clarified that phospholipase A₂ purified from human inflammatory sites very well resembles phospholipase A₂ purified from rat inflammation region in the activity and structure. In other words, both enzymes showed high specificity to phosphatidylethanolamin. On the contrary, the phospholipase A₂ purified from pancreas does not exhibit
35 such substrate specificity. The sequence of 34 amino acids on the N-terminal was compared and resultantly, the homology between the phospholipase A₂ purified from rat inflammation sites and the phospholipase A₂ purified from human inflammation sites was 67 %, while it was only 45 % between the phospholipase A₂ purified from human inflammatory sites and the phospholipase A₂ purified from pancreas.

In the meantime, the inventors have found a protein fraction which specifically inhibits the
40 phospholipase A₂ purified from rat peritoneal exudate, in the peritoneal wash of the rats to which dexamethasone was given. The inventors purified the fraction to obtain proteins of about 35 kDa and 40 kDa and determined the amino acid sequence of individual N-terminals [Japanese Patent laid-open No. S 63-246397 (1988) (Japanese Patent Application No. S 62-79693 (1987))].

Further, the inventors obtained, as a protein specifically inhibiting phospholipase A₂ purified from
45 synovial fluid of humans with rheumatoid arthritis, a protein of about 33 kDa as an enzymatic degradation products of human complement C3 and determined the amino acid sequence of the N-terminal (Japanese Patent Laid-open No. H 2-91100 (1990) (Japanese Patent Application No. S 63-242556 (1988))).

These inhibitory proteins did not exhibit the inhibitory activity against the pancreas phospholipase A₂ at all. In other words, these inhibitory proteins are quite unique because they have activities which have
50 overcome the two above-mentioned problems and can be expected to manifest much more powerful anti-inflammatory action than that of the previously isolated phospholipase A₂-inhibitory protein.

The complete amino acid sequence of the protein inhibiting the phospholipase A₂ purified from inflammatory sites has not yet been elucidated. In addition, it is very extremely difficult to obtain a sufficient amount of the phospholipase A₂-inhibitory protein to evaluate the inflammatory action, when it is extracted and purified from the rat peritoneal wash or degradation products of human C3.

Meanwhile, according to the inventors, the N-terminal amino acid sequence of protein inhibitors of phospholipase A₂ purified from inflammatory sites, which has been purified from rat peritoneal wash, has extremely high homology with a part of the amino acid sequence of human complement C3 α chain and is presumably a protein similar to C3dg or C3d, a degradation product of C3.

5 It has been known that human complement C3 is cleaved stepwise by protease in serum into C3dg or C3d in the final stage. In addition, it has been reported that a high level of C3dg is detected in the synovial fluid of humans with rheumatoid arthritis by an immunochemical method and the values are correlated to the severity of the disease (Nydegger et al., J. Clin. Invest., 59, 862-868, 1977).

Previously, Japanese Patent Laid-open No. H 2-91100 (1990) described above is cited as a document
10 relating to the correlation between the complement C3 and the phospholipase A₂-inhibitory protein purified from inflammatory sites. The patent document, however, does not describe the complete amino acid sequence and the gene of the inhibitory protein at all.

Further, there is no description of the correlation between said inhibitory protein and C3, particularly C3dg, a degradation product of complement C3, and of the preparation process for said inhibitory protein
15 by enzymatic treatment of serum.

The object of the invention is to give the complete amino acid sequence of protein inhibitors of phospholipase A₂ purified from inflammatory sites clearly.

Another object of the invention is to clarify the DNA sequence coding the amino acid sequence of protein inhibitors of phospholipase A₂ purified from inflammatory sites and to provide the preparation
20 process for giving a large amount of the protein.

Disclosure of the Invention

The inventors have made intensified study, as considering the problems of prior arts, and reached the
25 present invention by finding that a protein which is coded with 1032 bases and composed of 344 amino acids, and another protein which is coded with 1047 bases and composed of 349 amino acids, have inhibitory activity against phospholipase A₂ purified from inflammatory sites, respectively.

Namely, the present invention is protein inhibitors of phospholipase A₂ purified from inflammatory sites wherein said inhibitors have the amino acid sequence shown in Fig. 1 or an amino acid sequence
30 physiologically equivalent thereto.

Further, the present invention is a production process for an inhibitory protein against phospholipase A₂ purified from inflammatory sites, said protein having the amino acid sequence given in Fig. 1 or amino acid sequences having physiologically equivalent thereto wherein said process comprises enzymatic treatment of mammalian serum.

35 The present invention is also a gene of the inhibitory protein against phospholipase A₂ purified from inflammatory sites wherein said protein has the amino acid sequence given in Fig. 1 or an amino acid sequence physiologically equivalent thereto.

Further, the present invention is an inhibitory protein against phospholipase A₂ purified from human inflammatory sites wherein said protein has the amino acid sequence given in Fig. 3 or an amino acid
40 sequence physiologically equivalent thereto.

The present invention is a production process for an inhibitory protein against phospholipase A₂ purified from inflammatory sites, said protein having the amino acid sequence given in Fig. 3 or an amino acid sequence physiologically equivalent thereto wherein said process comprises enzymatic treatment of human serum.

45 Finally, the present invention is also a gene which codes the inhibitory protein against phospholipase A₂ purified from human inflammatory sites wherein said protein having the amino acid sequence given in Fig. 3 or an amino acid sequence physiologically equivalent thereto.

Brief Description of the Drawings

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Fig. 1 gives the amino acid sequence of the rat inhibitory protein-rat against phospholipase A₂ purified from inflammatory sites (abbreviated to inhibitory protein-rat) according to the invention.

Fig. 2 gives an example of DNA base sequence including the gene of the inhibitory protein-rat.

Fig. 3 shows the amino acid sequence of human phospholipase A₂-inhibitory protein purified from
55 inflammatory site-human (abbreviated to inhibitory protein-human hereinafter) according to the present invention.

Fig. 4 shows an example of the DNA base sequence of the gene for the inhibitory protein-human according to the present invention.

Fig. 5 depicts an elution pattern of the inhibitory protein-rat in anion-exchange HPLC.

Fig. 6 depicts an elution pattern of the inhibitory protein-rat in gel filtration HPLC.

Fig. 7 depicts an elution pattern of the inhibitory protein-rat in anion-exchange HPLC.

Fig. 8 depicts an elution pattern of the inhibitory protein-rat in reversed phase HPLC.

5 Fig. 9 gives SDS-PAGE of the inhibitory protein-rat.

Fig. 10 shows the inhibitory activity of the inhibitory protein-rat according to the present invention.

Fig. 11 (including 11a and 11b) gives the inhibitory activity of the inhibitory protein-rat according to the present invention against a variety of phospholipase A₂.

Fig. 12 gives Western blotting results of untreated and 37° C-treated human serums.

10 Fig. 13 depicts an elution pattern (13a) and SDS-PAGE (13b) of the inhibitory protein-human in preparative anion-exchange HPLC.

Fig. 14 depicts an elution pattern (14a) and SDS-PAGE (14b) of the inhibitory protein-human in analytical anion-exchange HPLC.

15 Fig. 15 depicts an elution pattern (Fig. 15a) and SDS-PAGE (Fig. 15b) of the inhibitory protein-human in gel filtration HPLC.

Fig. 16 depicts an elution pattern (Fig. 16a) and SDS-PAGE (Fig. 16b) in reverse-phase HPLC.

Fig. 17 shows the inhibitory activity of the inhibitory protein-human according to the present invention.

Fig. 18 shows the strategy for determining the base sequence of the gene containing the inhibitory protein-rat according to the present invention.

20 Fig. 19 gives the amino acid sequence near the breakage site of rat C3 by factor I of human C3.

Fig. 20 depicts SDS-PAGE (Fig. 20a) and Western Blotting (Fig. 20b) of inhibitory protein-human according to the present invention.

Best Embodiment of the Invention

25

The protein inhibitors of phospholipase A₂ purified from inflammatory sites according to the present invention is a polypeptide having the sequence of 344 amino acids given in Fig. 1 or another one having the sequence of 349 amino acids given in Fig. 3, and other polypeptides are also included in the protein inhibitors of phospholipase A₂ purified from inflammatory sites according to the present invention, as long as they have a substantially equal level of biological activity to the inhibitory protein-human, even when the amino acid sequence of the inhibitory protein is partially substituted, deleted or inserted.

30

In the preparative process according to the present invention, the protein inhibitors of phospholipase A₂ purified from inflammatory sites are produced by enzymatic treatment of serum from human, rat or the like and the enzymatic treatment means direct treatment of serum at 37° C for 6 to 10 days or treatment of C3 purified from serum with Factor I by usual operations.

35

Further, the gene of protein inhibitors of phospholipase A₂ purified from inflammatory sites according to the present invention comprised the sequence of 1032 DNAs given in Fig. 2 or of 1047 DNA given in Fig. 4, and the single stranded DNA and the double stranded DNA consisting of the single stranded DNA and the complementally stranded DNA are also included in the scope of the present invention.

40

Other DNA are also included in the genes of protein inhibitors of phospholipase A₂ purified from inflammatory sites according to the present invention, as long as they code the amino acid sequence of the inhibitory protein, even when the DNA has a base sequence resulting from partial substitution, deletion or insertion of the bases in the inhibitory protein gene, or the DNA includes the above-stated base sequence or a part of the above-stated base sequence.

45

Then, the method according to the present invention will be illustrated for reaching the complete amino acid sequence of the protein inhibitors of phospholipase A₂ purified from inflammatory sites and the gene of the inhibitory protein of the invention.

50

The protein inhibitor of phospholipase A₂ purified from inflammatory sites of the invention is isolated and purified by heat-treating or enzymatically treating serum and subjecting the resultant sample to anion-exchange HPLC, gel filtration HPLC and reverse phase HPLC, as the activity of phospholipase A₂ purified from inflammatory sites is used as an index, as described by Vik [Vik, D.P. et al. J. Immunol., 134, 2571 (1985)].

55

The serum used in the invention is mammalian serum, particularly rat serum or human serum. Then, the sample which has been isolated and purified is determined its N-terminal amino acid sequence by the gas-phase protein sequencer (Applied Biosystem Co.).

The inventors had cloned a rat C3 cDNA, γ rC3/11 which harbored an about 2.7 kbp of insert DNA and was regarded to include the whole part of the gene coding the inhibitory protein according the present invention, which is presumably a protein analogue to human C3dg.

Hereupon, the base sequence of the clone was determined and the base sequence of the gene of the inhibitory protein was identified in the comparison with the N-terminal amino acid sequence of the inhibitory protein.

In the case of the human protein, the N-terminal amino acid sequence of the purified sample was determined to be identical to human C3dg. Thus, the complete base sequence of the gene of the inhibitory protein was determined on the basis of the base sequence of human C3 gene [de Bruijn et al., Proc. Natl. Acad. Sci. USA, 82, 708-712(1985)].

Then, the complete amino acid sequence of the inhibitory protein was determined from the entire base sequence.

The tests and measurement methods used in the present invention were as follows:

(1) Measurement of the inhibitory activity against phospholipase A₂ purified from inflammatory sites

The phospholipase A₂ which was isolated and purified from the peritoneal exudate of rats with peritonitis caused by casein or from the synovial fluid of the humans with rheumatoid arthritis and the substrate was ¹⁴C-phosphatidyl-ethanolamine (about 2,000 dpm/nmol. 1 mM) extracted from E. coli cultured in the presence of ¹⁴C-acetic acid.

As for the enzymatic reactions, an inhibitor sample and water were combined with 50 μ l of 0.5M Tris • HCl (pH 9.0), 25 μ l of 40mM CaCl₂ and 25 μ l of the substrate to adjust the total volume to 240 μ l. They were mixed, finally 10 μ l of an enzyme solution (0.1ng/ μ l) was added to the mixture to start the reactions at 37 °C for 10 minutes and the Dole's reagent was added (1.5 ml) to stop the reactions. According to the Dole's method, ¹⁴C-fatty acid was extracted and the radioactivity was measured with a liquid scintillation counter.

(a) SDS-polyacrylamide gel electrophoresis and Western blotting

One tenth volume of a dye solution (0.1% BPB, XC, 10% SDS) was added to a sample obtained from the enzymatic treatment or HPLC, they were heat-treated at 100 °C for 5 minutes, applied to 12.5 % polyacrylamide gel and electrophoresed in the presence of 1% SDS with 15 to 25 mA for 1.5 hours. For analysis under reductive conditions, 2-mercaptoethanol (2ME) was added to the sample. After electrophoresis, the mixture was stained with CBB to detect the protein.

For the western blotting after electrophoresis, the protein was transferred from the gel to a nitrocellulose filter using an electroblot apparatus (Millipore Co.). Sheep anti-human C3d serum (Dako), horseradish peroxidase-conjugated anti-sheep IgG antibody (Cappel) and 4-chloro-1-naphthol (Bio-Rad) as a substrate were used to detect the C3d band by the enzyme-linked immunostaining assay.

(3) Determination of N-terminal amino acid sequence of protein

A gas-phase protein sequencer (Applied Biosystem 477A) and HPLC (Applied Biosystem 120A) were employed.

In other words, 100 μ l of a sample dissolved in 0.1% TFA was applied to the gas-phase protein sequencer. The protein underwent automatic Edmann degradation and the PTH (phenylthiohydantoin) amino acid derivatives were analyzed in the form of amino acids by high performance liquid chromatography.

(4) Determination of C-terminal amino acid sequence of protein

About 150 μ g of a sample was dissolved in 100 μ l of 8M urea-125mM Tris • HCl buffer (pH 7.6), 2 μ l of 2-mercaptoethanol was added and they were allowed to stand at 37 °C for 4 hours. Then, 2 μ l of 4-vinylpyridine was added to start the reaction at 37 °C for 45 minutes. The sample was desalted by reverse phase HPLC (Vydac 218TP54) and freeze-dried. The sample was dissolved in 200 μ l of 8M urea-1% ammonium hydrogencarbonate solution, 600 μ l of 1% ammonium hydrogencarbonate solution was added to the solution, then 1 μ g of α -chymotrypsin was added to start the reaction at 37 °C for 16 hours. Since the C-terminal of the protein was anticipated to be Arg, chymotrypsin was used.

Acetic acid was added to the reaction mixture to adjust the pH to about 5, and applied to an anhydrochymotrypsin agarose column (TAKARA SHUZO) which was equilibrated with 50mM sodium acetate buffer (pH 5.0) containing 20 mM of CaCl₂.

The C-terminal fragment was recovered in the flow-through and its amino acid sequence was determined as in (3) to reveal the C-terminal amino acid sequence of the inhibitory protein.

The protein inhibitors of phospholipase A₂ purified from inflammatory sites according to the invention was elucidated on its complete amino acid sequence by the present invention, and can be produced by known methods of chemical synthesis, but the protein can be readily mass-produced by the recombinant DNA method.

5 The protein inhibitors of phospholipase A₂ purified from inflammatory sites will be utilized as an anti-inflammatory agent as well as in diagnosis of inflammatory diseases using an antibody obtained from the protein as an antigen or a kit for such diagnosis.

Example

10 The present invention will be illustrated by the following examples in more detail.

Example 1

15 Preparation of protein inhibitors of phospholipase A₂ purified from rat inflammatory sites (abbreviated to inhibitory protein-rat) and the measurement of the inhibitory activity

(A) Preparation of inhibitory protein-rat

(1) 0.1% of NaN₃ was added to rat serum (Japan Biomaterial) and treated at 37° C for 10 days. Protease inhibitor (20 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor, 0.5 mM EDTA) was added and the product was dialyzed twice against 3 liters of 20mM Tris • HCl (pH 7.5).

(2) the dialyzed sample from (1) (protein concentration: about 30 mg/ml) was diluted 20 times, centrifuged to remove insoluble substance and the target protein was isolated and purified as follows:

First, the sample was fractionated with a preparative anion-exchange HPLC column (TSK gel DEAE-5PW) (Fig. 5).

In Fig. 5, the continuous line draws the UV absorptions at 280 nm wavelength and the dotted line shows the gradient of NaCl concentration.

The elution was conducted with 20 mM Tris • HCl, pH 7.5-NaCl (0 → 0.35M) 2.5 ml/min, 2 min./tube. The column measured 21.5ø x 30 cm.

Then, the fractions Nr. 61 to 63 in which the 39kDa band was detected in SDS-PAGE in Fig. 5 were collected, concentrated, and further fractionated with a gel filtration HPLC column (TSK gel G3000SW) (Fig. 6). In Fig. 6, the continuous line draws UV absorptions at 280 nm and the arrow marks give the elution positions of molecular weight markers.

The elution conditions were 20 mM Tris • HCl, pH 7.5-1M NaCl, 0.5 ml/min. 2 min./tube.

Then, the fraction peaks Nr. 19 to 20 in which 39 kDa band was detected in SDS-PAGE in Fig. 6 were collected and purified with a preparative anion exchange HPLC column (TSK gel DEAE-5PW) (Fig. 7). In Fig. 7, the continuous line draws the UV absorptions at 280 nm and the dotted line shows the gradient of NaCl concentration.

The purification was conducted with 20 mM Tris • HCl, pH 7.5-NaCl (0 → 1.0M) 1.0 ml/min, 2 min./tube.

Finally, the main peak fraction Nr. 16 in Fig. 7 was further purified with a reversed phase HPLC column (Bio-Rad RP304) (Fig. 8). In Fig. 8, the continuous line draws the UV absorptions at 210 nm and the dotted line shows the gradient of the CH₃CN concentration.

The purification was conducted with 0.1% TFA/CH₃CN 0 - 80 %, 1 ml/min., 2 min./tube.

The inhibitory protein-rat, obtained as described above, (fraction Nr. 30) showed a single band in SDS-PAGE (estimated molecular weight: about 39 kDa) (Fig. 9). SDS-PAGE was carried out after the fractions 29 to 33 was concentrated 10 times, respectively.

(B) phospholipase A₂-inhibitory activity of the inhibitory protein-rat

The inhibitory protein according to the present invention, obtained in (A) (fraction Nr. 30 in reversed phase HPLC, protein concentration 40 μg/ml) was determined its inhibitory activity against phospholipase A₂ purified from inflammatory sites and other phospholipase A₂ by the procedures for determining the inhibitory activity as described above (rat platelet-secretory phospholipase A₂ was used as an enzyme. It is same as phospholipase A₂ purified from inflammatory sites).

(1) In order to examine the inhibitory activity of the inhibitory protein according to the present invention against rat-phospholipase A₂, various amounts of the inhibitory protein were examined (3, 6, 9, 12, 15 and 18 ng). The results are given in Fig. 10.

Fig. 10 clearly shows that the inhibitory protein according to the invention inhibits the phospholipase A₂ purified from rat inflammatory sites in a dose dependent manner. The amount of the inhibitory protein which is needed for inhibiting 50 % activity of 1 ng phospholipase A₂ was about 6 ng and IC₅₀ was 0.8×10^{-9} M.

5 (2) In addition to phospholipase A₂ purified from rat inflammatory site, phospholipase A₂ purified from synovial fluid from humans with rheumatoid arthritis, phospholipase A₂ originating from *Crotalus adamanteus* venom (a snake venom) (Sigma Co.), porcine pancreas phospholipase A₂ (Sigma Co.), *Naja naja* venom (a snake venom) phospholipase A₂ (Sigma Co.) were used to determine the inhibitory activity of the inhibitory protein according to the present invention. The results are shown in Fig. 11a and Fig. 11b, respectively.

10 As shown in Fig. 11, the inhibitory protein manifested an almost equal level of inhibitory activity against phospholipase A₂ purified from rat inflammatory site (O-O) and that purified from the synovial fluid of humans with rheumatoid arthritis (Δ-Δ), and *Crotalus adamanteus* venom phospholipase A₂ (□-□), but revealed no activity against porcine pancreas phospholipase A₂ (x - x) and *Naja naja* venom phospholipase A₂ (x x). Thus, it has been clarified that the inhibitory protein according to the present invention is specifically active against phospholipase A₂ purified from inflammatory sites.

15 (3) The inhibitory protein obtained in (A) revealed no change in its inhibitory activity, even when the substrate concentration in the assay system was changed from 0.1 to 0.35 mM. Further, the inhibitory protein was pre-incubated together with the enzymes before the assay, but no change was noticed in the activity.

20 These results indicated that the activity of the inhibitory protein is not caused by the interaction with the substrate.

(4) Consequently, it has been clearly shown that the inhibitory protein has almost the same molecular weight and the same level of activity as of protein inhibitors of phospholipase A₂ purified from inflammatory sites from rat peritoneal cavity.

Example 2

Preparation of protein inhibitors of phospholipase A₂ purified from human inflammatory sites (abbreviated to inhibitory protein-human) and the measurement of its inhibitory activity

30 (A) Preparation of inhibitory protein-human

(1) 30 ml of human serum was mixed with 0.1 % NaN₃ and incubated at 37° C for 10 days.

35 Treated and untreated samples were diluted 50 times, respectively, subjected to SDS-PAGE and transferred to filters by the western blotting method. Each filter was subjected to the enzyme-linked immunostaining method using anti-human C3d serum, and only more than 100 kDa band was detected in the untreated serum, while only 39 kDa band, in the serum treated at 37° C (Fig. 12). These results proved that the treatment at 37° C caused cleavage of C3 by enzyme in serum to produce C3dg.

Hereupon, protease inhibitor (20 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor, 0.5 mM EDTA) was added, and the mixture was dialyzed twice against 3 liters of 20 mM Tris · NaCl.

40 (2) The dialyzed sample obtained in (1) (protein concentration: about 30 mg/ml) was diluted 20 times, insoluble substances were removed by centrifugation and the target protein was isolated and purified as follows:

45 First, the sample was fractionated with an preparative anion exchange HPLC column (TSK gel DEAE-5PW) (Fig. 13). In Fig. 13a, the continuous line depicts the absorption at 280 nm, while the dotted line gives the NaCl concentration gradient. The elution was conducted under the conditions of 20 mM Tris · HCl, pH 7.5-NaCl (0 → 0.35 M), 2.5 ml/mlin, 2 min/tube. The column measured 21.5 mmø X 30 cm.

50 Then, fractions Nr. 52 to 54 in which 39 kDa band was detected in SDS-PAGE among fractions given in Fig. 13b were collected, diluted 4 times and purified with a preparative anion-exchange HPLC column (TSK gel DEAE-5PW) (Fig. 14). In Fig. 14a, the continuous line draws the UV absorption at 280 nm, the dotted line depicts the gradient of NaCl concentration, and the bar graph shows the inhibitory activity against phospholipase A₂ purified from human inflammatory sites.

The purification conditions were 20 mM Tris · HCl, pH 7.5-1M NaCl (0 → 1.0M), 1.0 ml/min. 2 min./tube.

55 Then, the fraction Nr. 13 in which the 39 kDa band was detected in SDS-PAGE in Fig. 14b and the inhibitory activity against phospholipase A₂ purified from human inflammatory sites was strongest was collected and additionally fractionated with a gel filtration HPLC column (TSK gel G3000SW) (Fig. 15). In Fig. 15a, the continuous line draws the UV absorption at 280 nm, and the bar graph shows the inhibitory activity against phospholipase A₂ purified from human inflammatory sites.

The elution conditions were 20 mM Tris • HCl, pH 7.5-1M NaCl 0.5 ml/min. 2 min./tube.

Finally, fractions Nr. 20 and 21 in which the 39 kDa band was detected in SDS-PAGE in Fig. 15b and the inhibitory activity against phospholipase A₂ purified from human inflammatory sites was strongest were collected and additionally purified with a reverse phase HPLC column (Bio-Rad RP304) (Fig. 16). In Fig. 16a, the continuous line draws the UV absorption at 210 nm and the dotted line depicts the gradient of CH₃CN concentration.

The purification conditions were 0.1% TFA/CH₃CN 0 to 80 %, 1 ml/min, 2 min./tube.

As shown in SDS-PAGE in Fig. 16b, the inhibitory protein-human of molecular weight 39 kDa was eluted in fraction Nr. 30, but it has been found that the protein is contaminated with a small amount of another protein which was mainly eluted in fraction Nr. 23 and may be albumin. But, albumin does not have strong inhibitory activity against phospholipase A₂, and fraction Nr. 23 showed no phospholipase A₂-inhibitory activity, either. Thus, it was concluded that they cause no influence on the activity determination of the inhibitory protein.

15 (B) phospholipase A₂-inhibitory activity of inhibitory protein-human

The inhibitory protein-human according to the present invention, obtained in (A), (fraction Nr. 30 in reversed phase HPLC, protein concentration 15 ng/ μ l) was determined its inhibitory activity against phospholipase A₂ purified from human inflammatory site and other phospholipase A₂ according to the method described in Example 1.

The results are given in Fig. 17 and Table 1.

Table 1: Comparison of inhibitory activity of the inhibitory protein of the invention against various phospholipases A₂

source of phospholipase A ₂	the inhibitory activity (%) [*] against phospholipase A ₂
synovial fluid from human with rheumatoid arthritis	47.4
rat platelet (purified from rat inflammatory site)	22.0
porcine pancreas	6.5

* inhibitory activity when about 15 ng of inhibitory protein-human was added to 1 ng of phospholipase A₂.

It is evident from Fig. 17 that the inhibitory protein according to the present invention inhibits phospholipase A₂ purified from human inflammatory site in a dose dependent manner. The amount of the inhibitory protein needed for inhibiting 1 ng of phospholipase A₂ purified from human inflammatory sites was about 50 ng. As shown clearly in Table 1, the inhibitory protein inhibited phospholipase A₂ purified from rat inflammatory sites, but not phospholipase A₂ purified from porcine pancreas.

As a result of these facts, it can be concluded that the inhibitory protein according to the present invention has a specific inhibitory activity against phospholipase A₂ purified from human inflammatory sites.

Example 3

Identification of phospholipase A₂-inhibitory protein originating from rat inflammatory sites

55 (A) Determination of N-terminal amino acid sequence in the inhibitory protein-rat

The 39 kDa inhibitory protein, which was isolated and purified in Example 1 (A), was determined its N-

terminal amino acid sequence by means of the gas-phase protein sequencer 477A (Applied Biosystem Co.). The sequence was as follows:

NH₂-Glu-Asp-Val-Pro-Ala-Ala-Asp-Leu-Ser-

This sequence was identical completely to the N-terminal sequence of C3dg which is deduced from the
 5 base sequence of rat C3 cDNA which will be stated later and was also identical completely to the N-terminal amino acid sequence of the protein inhibitors of phospholipase A₂ purified from peritoneal exudate of a rat treated with dexamethasone.

(B) The C-terminal fragment of the inhibitory protein was determined its amino acid sequence as in (A) and
 10 the following sequence was obtained:

Gln-Thr-Asp-Val-Pro-Asp-His-Lys-Asp-Leu-Asn-Met-Asp-Val-Ser-Leu-His-Leu-Pro-Ser-Arg.

This sequence was completely identical to the C-terminal sequence of C3dg which is anticipated from the base sequence of rat C3 cDNA which will be stated later.

15 (C) Western blotting of inhibitory protein-rat

The inhibitory protein-rat of about 39 kDa molecular weight, which was isolated and purified in Example 1 (A), was subjected to SDS-PAGE in a usual manner, and transferred to a nitrocellulose membrane filter using an electroblotting apparatus (Milipore Co.) according to the protocol proposed by Milipore. The filter
 20 was stained by the enzyme-linked immunostaining method using rabbit anti-human C3d serum (DAKOPATTS) as a primary antibody, anti-rabbit Ig G horseradish peroxidase-conjugated sheep Ig G fraction (CAPPEL) as a secondary antibody, and 4-chloro-1-naphthol (Bio-Rad) as a substrate.

As a result, the inhibitory protein specifically reacted with anti-human C3d serum.

From (A), (B) and (C), the inhibitory protein according to the present invention was identified as rat
 25 C3dg.

Example 4

cDNA cloning of protein inhibitors of phospholipase A₂ purified from inflammatory sites

30 (A) Preparation of mouse C3 cDNA segment

The Hind III digest of plasmid pFC4/5.4 containing mouse C3 cDNA segment (J. B. C. 260, 10936, 1986) was subjected to the low-melting agarose gel electrophoresis to separate the 2.2 kbp segment. Further, Stu I digest was subjected to similar operations to give 1.8 kbp segment.

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(B) Screening of cDNA

Screening was performed using rat liver cDNA λ gt 11 library (Clone Tech Co.) according to the manual of the experimental protocol by the Clone Tech.

40 In other words, E. coli 1090 line infected with λ gt 11 phage was cultured at 37° C for 5 hours and the resultant about 50,000 transformants were replicated to a nylon filter membrane, dipped in 0.5 M NaOH-1.5 M NaCl to modify the DNA, and neutralized with Tris • HCl buffer containing 1.5 M NaCl-1 mM EDTA (pH 7.2). Then, the filter was air-dried, and the DNA was immobilized to the filter by irradiating with ultraviolet rays for 3 minutes using a trans-illuminator. The mouse C3 cDNA obtained in (A) was labeled with ³²P, and
 45 used as a screening probe.

The transformant groups on the examined filter were screened by hybridization with the probe at 65° C and the hybridization was judged by the autoradiography and 7 transformants were found to be positively cloned among about 50,000 transformants. These positive clones were named λ r C3/11 - 17.

50 These clones were digested with EcoRI, then agarose gel electrophoresis was conducted to analyze the chain length of the inserted gene segment according to southern hybridization. The inserted DNA of λ r C3/11 was longest among these seven clones and it was found to have the insertion gene segment of 2.7 kbp total length which is digested into 0.7 kbp and 2.0 kbp segments by EcoRI.

(C) Determination of base sequence of rat C3 cDNA

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After phage DNA of λ r C3/11 was extracted, the DNA was digested with EcoRI to give 0.7 kbp and 2.0 kbp DNA segments. These segments were inserted into the EcoRI sites of M13mp19 (TAKARA SHUZO). The 0.7 kbp segment was further digested with BamHI into 0.1 kbp segment and 0.6 kbp segment, and

these segments were inserted into the mp19 to determine the base sequence from both ends.

The sequence was analyzed according to the 7-DEAZA technique [Mizusawa, et. al., Nucleic Acid Res., 14, 1319 (1986)]. The 2.0 kbp segment was analyzed according to the technique developed by Hood et al in which the sequence analysis is put forward, as the primers designed from the determined region are synthesized [Hood, et. al., Anal. Biochem., 154, 353 (1986)].

The strategy for determining the base sequence of λ r C3/11 is given in Fig. 18. In the Figure, the uppermost numerals are the base numbers at the breakage points based on the framed part of rat C3dg, EcoRI and others are the names of restriction enzymes and the numerals in parentheses are the base number on the cleavage site or the starting site for determination of base sequence. The horizontal arrows give the direction and range for determining base sequence with restriction enzymes and the primer, respectively.

Example 5

Determination of the base sequence of the gene coding protein inhibitors of phospholipase A₂ purified from rat inflammatory sites and the amino acid sequence of the protein

(1) Determination of N-terminal and C-terminal

Fig. 19 shows the part corresponding to the amino acid sequence in the neighborhood of the breakage sites by human C3 factor I, among the rat C3 amino acid sequence presumed from the base sequence (C) of the C3 cDNA clone which has been elucidated in Example 4.

Human C3 is cleaved by factor I between ²⁸³Arg-²⁸⁴Glu in the α chain, but the corresponding site shifts to Gln-Gly in rat C3 and Arg-Glu exists at the site five residues after Gln-Gly. And, the N-terminal amino acid sequence of the fragment formed by cleavage at this site was completely identical to the N-terminal amino acid sequence of the inhibitory protein according to the invention, which was purified from rat serum.

Accordingly, it is thought that rat C3 is cleaved at this site by an enzyme having similar activity to human factor I.

In the meantime, ⁶³²Arg-⁶³³Ser and ⁶⁴⁹Arg-⁶⁵⁰Ser in human C3 α chain were conserved in rat C3, too. Further, the C-terminal amino acid sequence of the fragment formed by cleavage at this site was completely identical to the C-terminal amino acid sequence of the inhibitory protein according to the present invention, purified from rat serum. As in human C3, rat C3 is presumably cleaved at these sites by factor I and the C-terminal of the inhibitory protein according to the present invention was identical to the C-terminal of human C3dg.

(2) The complete base sequence of the gene of protein inhibitors of phospholipase A₂ purified from rat inflammatory sites and deduced complete amino acid sequence

On the basis on these results, the complete amino acid sequence of the protein inhibitor of phospholipase A₂ purified from inflammatory sites and the complete base sequence of the gene according to the present invention can be given in Fig. 1 and Fig. 2. The protein inhibitor of phospholipase A₂ purified from inflammatory sites has been found to be a protein which is coded with 1032 bases and is composed of 344 amino acids.

Example 6

Determination of the complete base sequence of the gene of protein inhibitors of phospholipase A₂ purified from human inflammatory sites and the complete amino acid sequence of the inhibitory protein

(A) Identification of inhibitory protein-human

(1) Determination of the N-terminal amino acid sequence of inhibitory protein-human

The N-terminal amino acid sequence was determined in the same manner as described above on the inhibitory protein of about 39 kDa molecular weight which was isolated and purified in Example 2(A) (fraction Nr. 30 in the reverse phase HPLC). As a result, the following sequence was given:

NH₂-Glu-Gly-Val-Asn-Lys-Glu-Asp-Ile-Pro-Pro-

This sequence was completely identical to the N-terminal amino acid sequence of human C3dg.

(2) Immunochemical analysis of inhibitory protein-human

After the inhibitory protein of about 39 kDa molecular weight which was isolated and purified in Example 2(A) (fraction Nr. 30 in reverse phase HPLC) was subjected to SDS-PAGE, the protein was transferred to a filter by the western blotting method and detected by the enzyme-linked immunostaining method using anti-human C3d serum. As a result, the inhibitory protein according to the present invention reacted with anti-
 5 human C3d serum sepcifically. Fig. 20a gives the SDS-PAGE, while Fig. 20b shows the western blotting.

The results from (1) and (2) evidently reveals that the inhibitory protein according to the present invention is human C3dg.

(B) The complete base sequence of the gene of protein inhibitors of phospholipase A₂ purified from human
 10 inflammatory sites (C3 dg) and the complete amino acid sequence of the inhibitory protein

cDNA cloning of human C3 was already established and the complete base sequence and the complete amino acid sequence deduced therefrom were determined. Further, the part coding C3dg was also decided in the C3 gene [de Bruijn et al., Proc. Natl. Acad. Sci. USA, 82, 708-712 (1985)].

15 The results described above evidently show that the protein inhibitors of phospholipase A₂ purified from human inflammatory sites is human C3dg. Accordingly, the base sequence of the inhibitory protein of this invention is presumed to be completely identical to the part coding C3dg in the entire base sequence of the human C3 gene.

Consequently, it was judged that the complete amino acid sequence of the protein inhibitor of
 20 phospholipase A₂ purified from human inflammatory sites and the complete base sequence of the protein inhibitor of phospholipase A₂ purified from human inflammatory sites of the invention are those given in Fig. 3 and Fig. 4, respectively. The protein inhibitors of phospholipase A₂ purified from inflammatory sites was found to be a protein which is coded with 1047 bases and comprises 349 amino acids.

25 Claims

1. Protein inhibitors of phospholipase A₂ purified from inflammatory sites wherein said inhibitors have the amino acid sequence given in Fig. 1 or an amino acid sequence physiologically equivalent thereto.
- 30 2. Protein inhibitors of phospholipase A₂ purified from inflammatory sites of claim 1 wherein said amino acid sequence comprises a part of the one given in Fig. 1 or a part of an amino acid sequence physiologically equivalent thereto.
3. Protein inhibitors of phospholipase A₂ purified from inflammatory sites of claim 1 or claim 2 wherein
 35 said protein originates from rat serum.
4. A process for preparation of protein inhibitors of phospholipase A₂ purified from inflammatory sites of claim 1 wherein said process comprises enzymati treatment of serum of a mammalian animal.
- 40 5. A process for preparation of protein inhibitors of phospholipase A₂ purified from inflammatory sites of claim 4 wherein the mammalian animal is rat.
6. A gene of protein inhibitors of phospholipase A₂ purified from inflammatory sites of claim 1.
- 45 7. A gene of protein inhibitors of phospholipase A₂ purified from inflammatory sites of claim 6 wherein the gene comprises the base sequence given in Fig. 2.
8. A gene of protein inhibitors of phospholipase A₂ purified from inflammatory sites of claim 6 or claim 7 wherein said gene originates from rat.
- 50 9. Protein inhibitors of phospholipase A₂ purified from inflammatory sites wherein said inhibitors have the amino acid sequence given in Fig. 3 or an amino acid sequence physiologically equivalent thereto.
10. Protein inhibitors of phospholipase A₂ purified from inflammatory sites of claim 9 wherein said amino
 55 acid sequence comprises a part of the one given in Fig. 3 or a part of an amino acid sequence physiologically equivalent thereto.

11. A process for preparation of phospholipase A₂-inhibitory protein of claim 9 wherein said process

comprises enzymatic treatment of human serum.

12. A gene of protein inhibitors of phospholipase A₂ purified from inflammatory sites of claim 9.

5 13. A gene of protein inhibitors of phospholipase A₂ purified from inflammatory sites of claim 12 wherein said gene comprises the base sequence given in Fig. 4.

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FIG 1-1

Glu Asp Val Pro Ala Ala Asp Leu Ser Asp Gln Val 12

Pro Asp Thr Asp Ser Glu Thr Arg Ile Leu Leu Gln

Gly Thr Pro Val Ala Gln Met Ala Glu Asp Ala Val

Asp Gly Glu Arg Leu Lys His Leu Ile Val Thr Pro

Ser Gly Cys Gly Glu Glu Asn Met Ile Gly Met Thr 60

Pro Thr Val Ile Ala Val His Tyr Leu Asp Gln Thr

Glu Gln Trp Glu Lys Phe Gly Leu Glu Lys Arg Gln

Glu Ala Leu Glu Leu Ile Lys Lys Gly Tyr Thr Gln

Gln Leu Ala Phe Lys Gln Pro Ser Ser Ala Tyr Ala 108

Ala Phe Asn Asn Arg Pro Pro Ser Thr Trp Leu Thr

Ala Tyr Val Val Lys Val Phe Ser Leu Ala Ala Asn

Leu Ile Ala Ile Asp Ser Gln Val Leu Cys Gly Ala

Val Lys Trp Leu Ile Leu Glu Lys Gln Lys Pro Asp 156

Gly Val Phe Gln Glu Asp Gly Pro Val Ile His Gln

FIG 1-2

Glu Met Ile Gly Gly Phe Arg Asn Thr Lys Glu Ala 180
Asp Val Ser Leu Thr Ala Phe Val Leu Ile Ala Leu
Gln Glu Ala Arg Asp Ile Cys Glu Gly Gln Val Asn
Ser Leu Pro Gly Ser Ile Asn Lys Ala Gly Glu Tyr
Leu Glu Ala Ser Tyr Leu Asn Leu Gln Arg Pro Tyr 228
Thr Val Ala Ile Ala Gly Tyr Ala Leu Ala Leu Met
Asn Lys Leu Glu Glu Pro Tyr Leu Thr Lys Phe Leu
Asn Thr Ala Lys Asp Arg Asn Arg Trp Glu Glu Pro
Gly Gln Gln Leu Tyr Asn Val Glu Ala Thr Ser Tyr 276
Ala Leu Leu Ala Leu Leu Leu Leu Lys Asp Phe Asp
Ser Val Pro Pro Val Val Arg Trp Leu Asn Glu Gln
Arg Tyr Tyr Gly Gly Gly Tyr Gly Ser Thr Gln Ala
Thr Phe Met Val Phe Gln Ala Leu Ala Gln Tyr Gln 324
Thr Asp Val Pro Asp His Lys Asp Leu Asn Met Asp

FIG 1-3

Val Ser Leu His Leu Pro Ser Arg

344

FIG 2-1

GAG GAT GTA CCT GCA GCA GAC CTC AGT GAC CAA GTG 36
CCA GAC ACA GAT TCT GAG ACC AGA ATT CTC CTG CAA
GGG ACC CCG GTG GCT CAG ATG GCC GAC GAG GCT GTG
GAC GGG GAG CGG CTG AAA CAC CTG ATC GTG ACC CCC
TCT GGC TGT GGG GAG CAG AAC ATG ATT GGC ATG ACA 180
CCC ACG GTC ATT GCA GTA CAC TAT CTG GAT CAG ACC
GAA CAG TGG GAG AAA TTC GGC CTA GAG AAG AGG CAA
GAA GCT CTG GAG CTC ATC AAG AAA GGG TAC ACC CAG
CAG CTG GCT TTC AAA CAG CCC AGC TCT GCC TAT GCT
GCC TTC AAC AAC CGG CCT CCC AGC ACC TGG CTG ACA 360
GCC TAT GTG GTC AAG GTC TTC TCT CTG GCT GCC AAC
CTC ATC GCC ATC GAC TCT CAG GTC CTG TGT GGG GCT
GTC AAA TGG CTG ATT CTG GAG AAA CAG AAG CCA GAT
GGT GTC TTT CAG GAG GAC GGA CCA GTG ATT CAC CAA 504

FIG 2-2

GAA ATG ATT GGT GGC TTC CGG AAC ACC AAG GAG GCA 540

GAT GTG TCG CTT ACA GCC TTT GTC CTC ATC GCA CTG

CAG GAA GCC AGA GAT ATC TGT GAG GGG CAG GTC AAC

AGC CTT CCC GGG AGC ATC AAC AAG GCA GGG GAG TAT

CTT GAA GCC AGT TAC CTG AAC CTG CAG AGA CCA TAC

ACA GTA GCC ATT GCT GGG TAT GCC CTG GCC CTG ATG 720

AAC AAA CTG GAG GAA CCT TAC CTC ACC AAG TTT CTG

AAC ACA GCC AAA GAT CGG AAC CGC TGG GAG GAG CCT

GGC CAG CAG CTC TAC AAT GTG GAG GCC ACC TCC TAC

GCC CTC CTG GCC CTG CTG CTG CTG AAA GAC TTT GAC

TCT GTG CCT CCT GTG GTG CGC TGG CTC AAC GAG CAA 900

AGA TAC TAC GGA GGT GGC TAT GGC TCC ACG CAG GCT

ACC TTC ATG GTA TTC CAA GCC TTG GCT CAA TAC CAA

ACA GAT GTC CCT GAC CAC AAG GAC TTG AAC ATG GAT 1008

FIG 2-3

GTG TCC CTG CAC CTC CCC AGC CGC

1032

FIG 3-1

Glu Gly Val Gln Lys Glu Asp Ile Pro Pro Ala Asp 12
Leu Ser Asp Gln Val Pro Asp Thr Glu Ser Glu Thr
Arg Ile Leu Leu Gln Gly Thr Pro Val Ala Gln Met
Thr Glu Asp Ala Val Asp Ala Glu Arg Leu Lys His
Leu Ile Val Thr Pro Ser Gly Cys Gly Glu Gln Asn 60
Met Ile Gly Met Thr Pro Thr Val Ile Ala Val His
Tyr Leu Asp Glu Thr Glu Gln Trp Glu Lys Phe Gly
Leu Glu Lys Arg Gln Gly Ala Leu Glu Leu Ile Lys
Lys Gly Tyr Thr Gln Gln Leu Ala Phe Arg Gln Pro 180
Ser Ser Ala Phe Ala Ala Phe Val Lys Arg Ala Pro
Ser Thr Trp Leu Thr Ala Tyr Val Val Lys Val Phe
Ser Leu Ala Val Asn Leu Ile Ala Ile Asp Ser Gln

FIG 3-2

Val Leu Cys Gly Ala Val Lys Trp Leu Ile Leu Glu 156
Lys Gln Lys Pro Asp Gly Val Phe Gln Glu Asp Ala
Pro Val Ile His Gln Glu Met Ile Gly Gly Leu Arg
Asn Asn Asn Glu Lys Asp Met Ala Leu Thr Ala Phe
Val Leu Ile Ser Leu Gln Glu Ala Lys Asp Ile Cys 204
Glu Glu Gln Val Asn Ser Leu Pro Gly Ser Ile Thr
Lys Ala Gly Asp Phe Leu Glu Ala Asn Tyr Met Asn
Leu Gln Arg Ser Tyr Thr Val Ala Ile Ala Gly Tyr
Ala Leu Ala Gln Met Gly Arg Leu Lys Gly Pro Leu 252
Leu Asn Lys Phe Leu Thr Thr Ala Lys Asp Lys Asn
Arg Trp Glu Asp Pro Gly Lys Gln Leu Tyr Asn Val
Glu Ala Thr Ser Tyr Ala Leu Leu Ala Leu Leu Gln

FIG 3-3

Leu Lys Asp Phe Asp Phe Val Pro Pro Val Val Arg 300
 Trp Leu Asn Glu Gln Arg Tyr Tyr Gly Gly Gly Tyr
 Gly Ser Thr Gln Ala Thr Phe Met Val Phe Gln Ala
 Leu Ala Gln Tyr Gln Lys Asp Ala Pro Asp His Gln
 Glu Leu Asn Leu Asp Val Ser Leu Gln Leu Pro Ser 348
 Arg 349

FIG 4-1

GAA GGA GTG CAG AAA GAG GAC ATC CCA CCT GCA GAC 36
CTC AGT GAC CAA GTC CCG GAC ACC GAG TCT GAG ACC
AGA ATT CTC CTG CAA GGG ACC CCA GTG GCC CAG ATG
ACA GAG GAT GCC GTC GAC GCG GAA CGG CTG AAG CAC
CTC ATT GTG ACC CCC TCG GGC TGC GGG GAA CAG AAC 180
ATG ATC GGC ATG ACG CCC ACG GTC ATC GCT GTG CAT
TAC CTG GAT GAA ACG GAG CAG TGG GAG AAG TTC GGC
CTA GAG AAG CGG CAG GGG GCC TTG GAG CTC ATC AAG
AAG GGG TAC ACC CAG CAG CTG GCC TTC AGA CAA CCC
AGC TCT GCC TTT GCG GCC TTC GTG AAA CGG GCA CCC 360
AGC ACC TGG CTG ACC GCC TAC GTG GTC AAG GTC TTC
TCT CTG GCT GTC AAC CTC ATC GCC ATC GAC TCC CAA
GTC CTC TGC GGG GCT GTT AAA TGG CTG ATC CTG GAG 468

FIG 4-2

AAG CAG AAG CCC GAC GGG GTC TTC CAG GAG GAT GCG 504
CCC GTG ATA CAC CAA GAA ATG ATT GGT GGA TTA CGG
AAC AAC AAC GAG AAA GAC ATG GCC CTC ACG GCC TTT
GTT CTC ATC TCG CTG CAG GAG GCT AAA GAT ATT TGC
GAG GAG CAG GTC AAC AGC CTG CCA GGC AGC ATC ACT
AAA GCA GGA GAC TTC CTT GAA GCC AAC TAC ATG AAC
CTA CAG AGA TCC TAC ACT GTG GCC ATT GCT GGC TAT 720
GCT CTG GCC CAG ATG GGC AGG CTG AAG GGG CCT CTT
CTT AAC AAA TTT CTG ACC ACA GCC AAA GAT AAG AAC
CGC TGG GAG GAC CCT GGT AAG CAG CTC TAC AAC GTG
GAG GCC ACA TCC TAT GCC CTC TTG GCC CTA CTG CAG
CTA AAA GAC TTT GAC TTT GTG CCT CCC GTC GTG CGT 900
TGG CTC AAT GAA CAG AGA TAC TAC GGT GGT GGC TAT 936

FIG 4-3

GGC TCT ACC CAG GCC ACC TTC ATG GTG TTC CAA GCC 972

TTG GCT CAA TAC CAA AAG GAC GCC CCT GAC CAC CAG

GAA CTG AAC CTT GAT GTG TCC CTC CAA CTG CCC AGC 104

CGC 1047

FIG 5

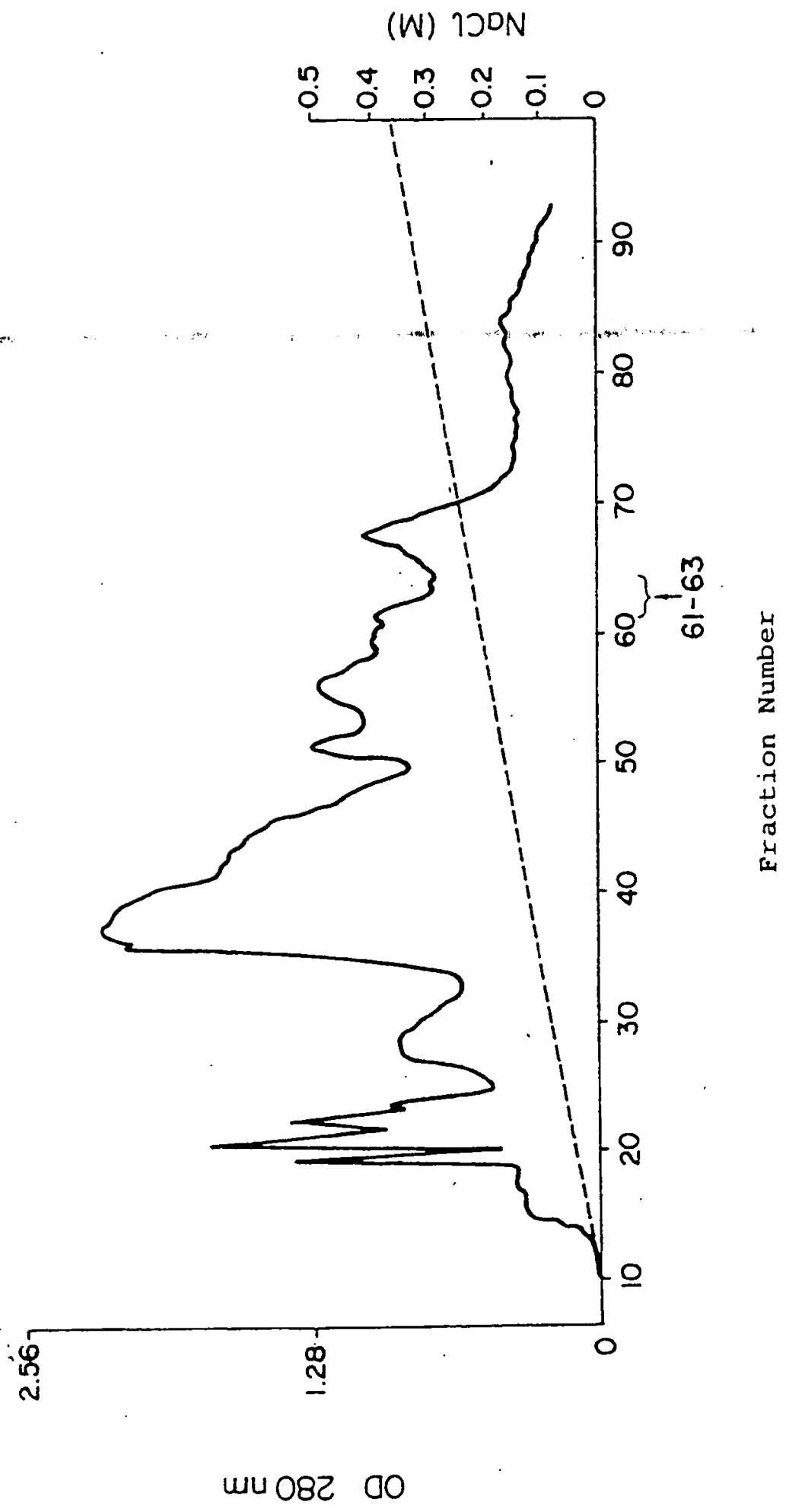


FIG 6

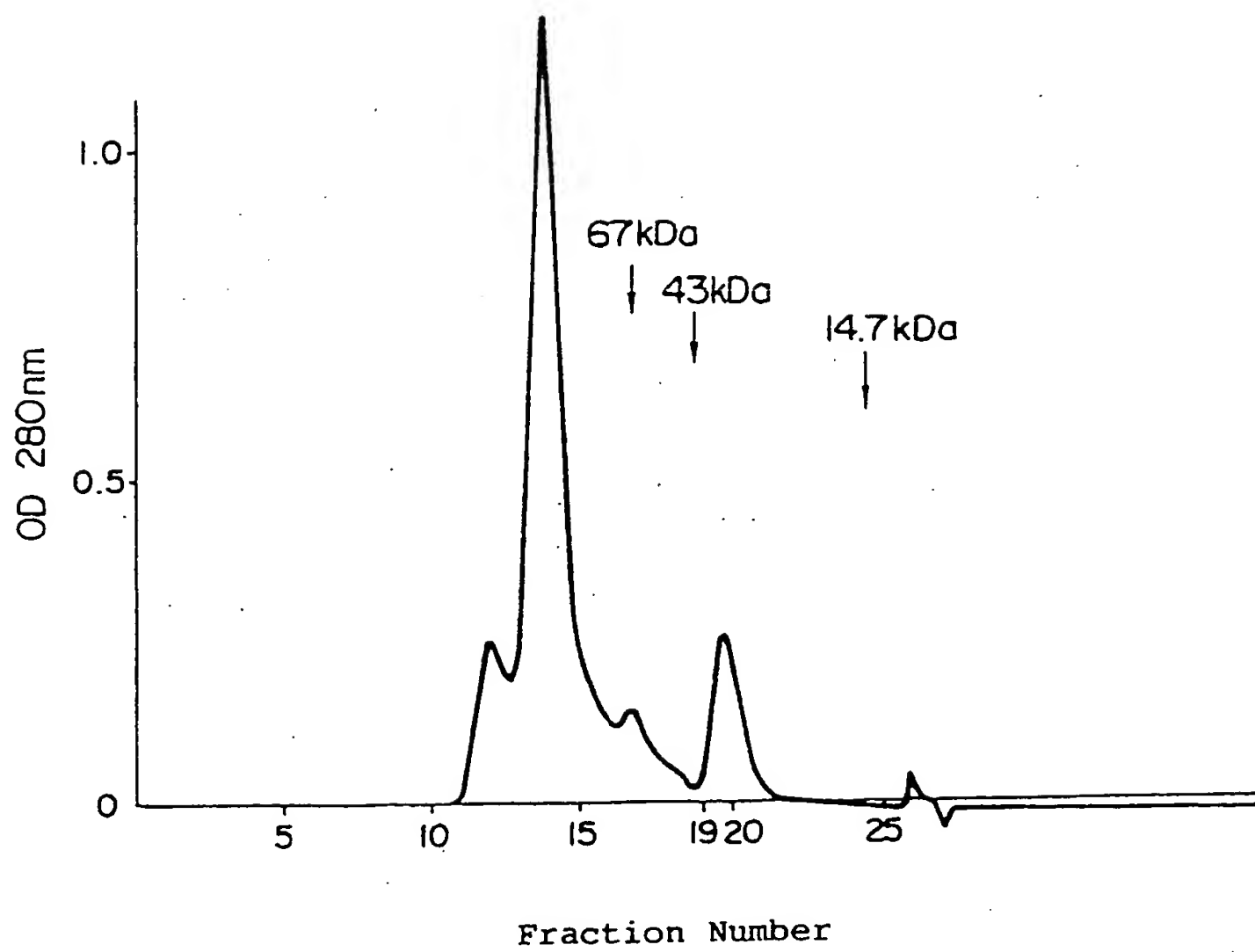


FIG 7

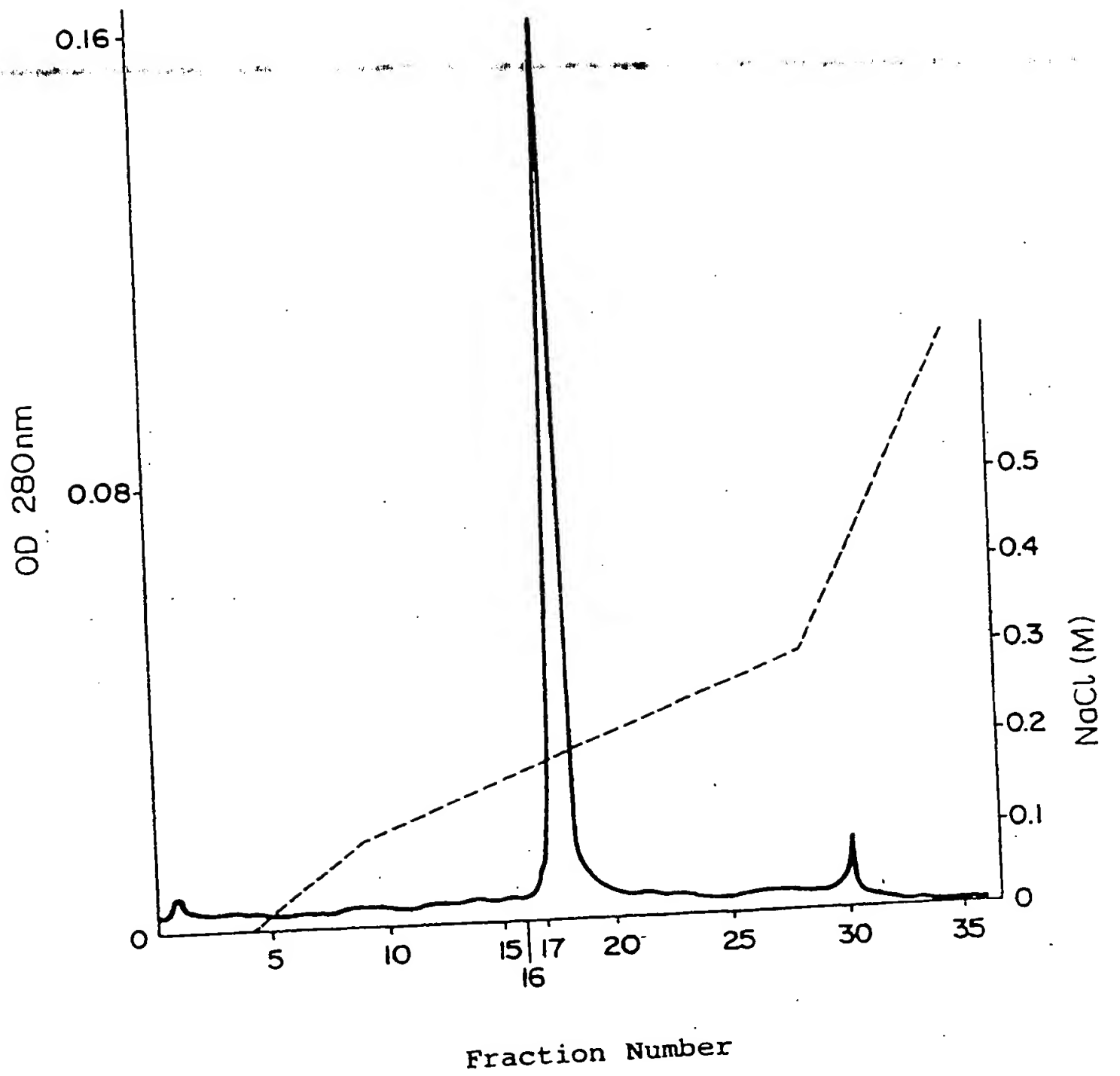


FIG 8

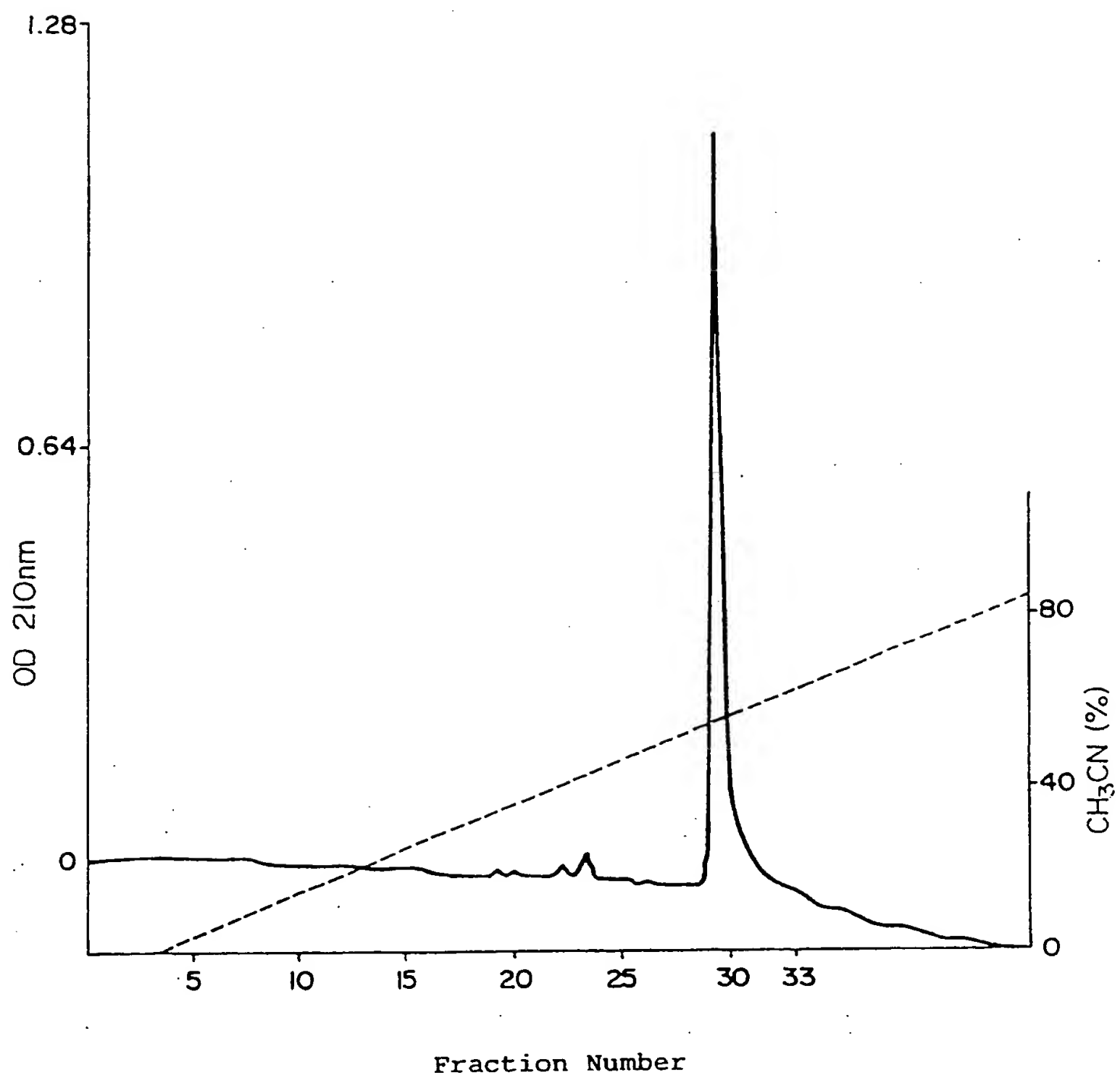


FIG 9

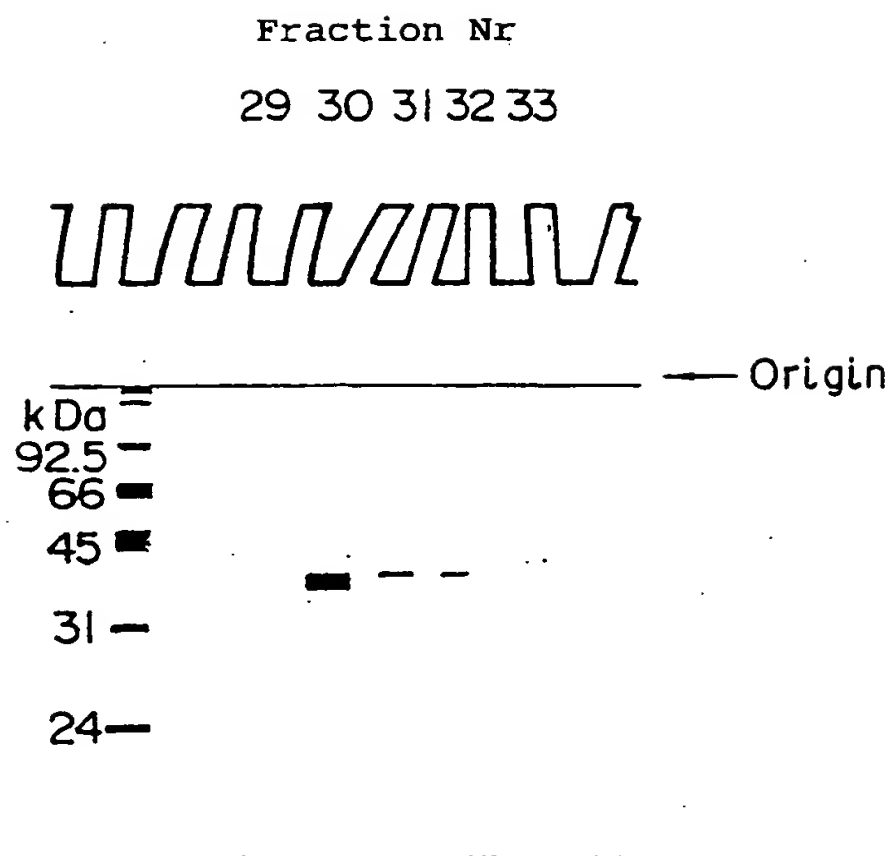


FIG 10

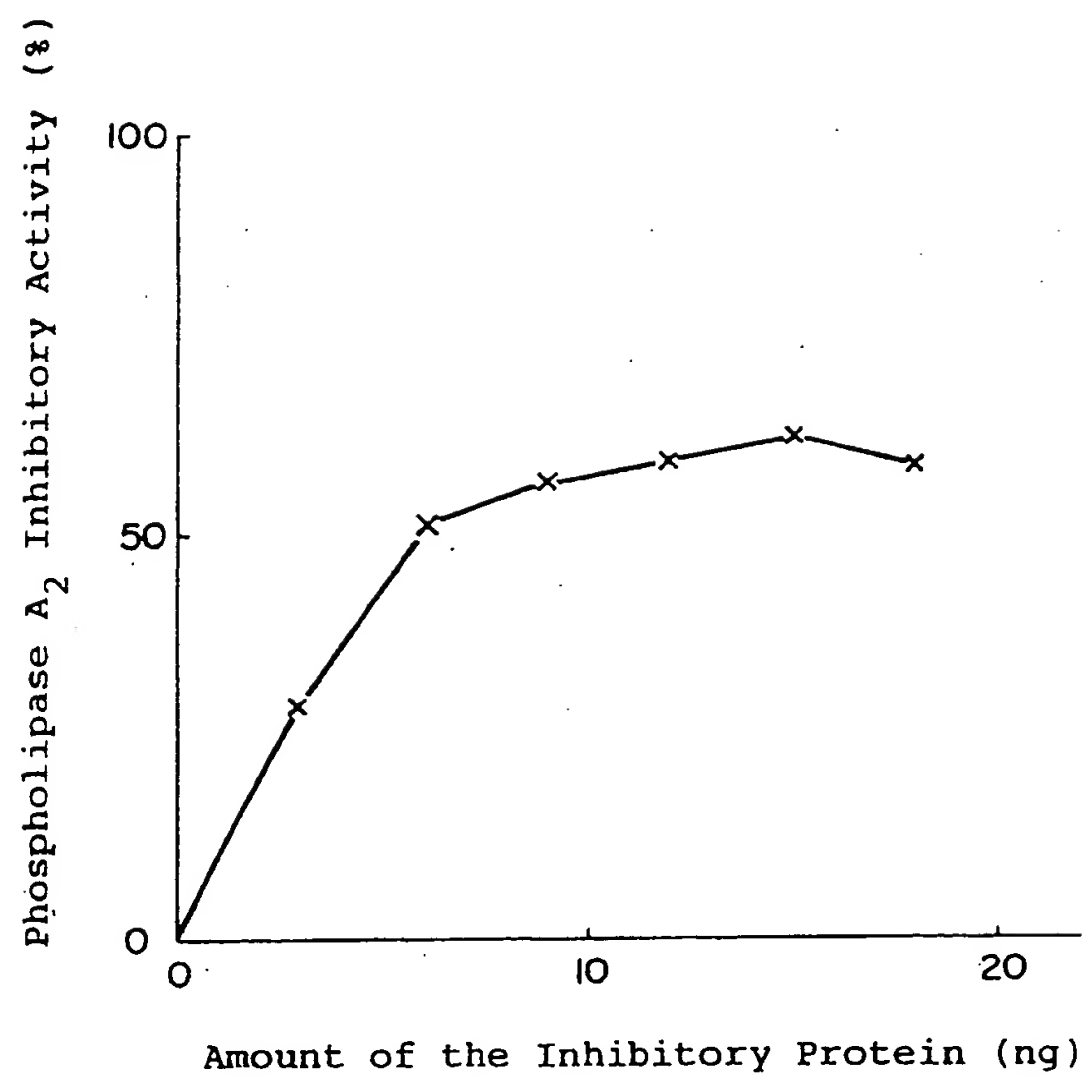


FIG 11a

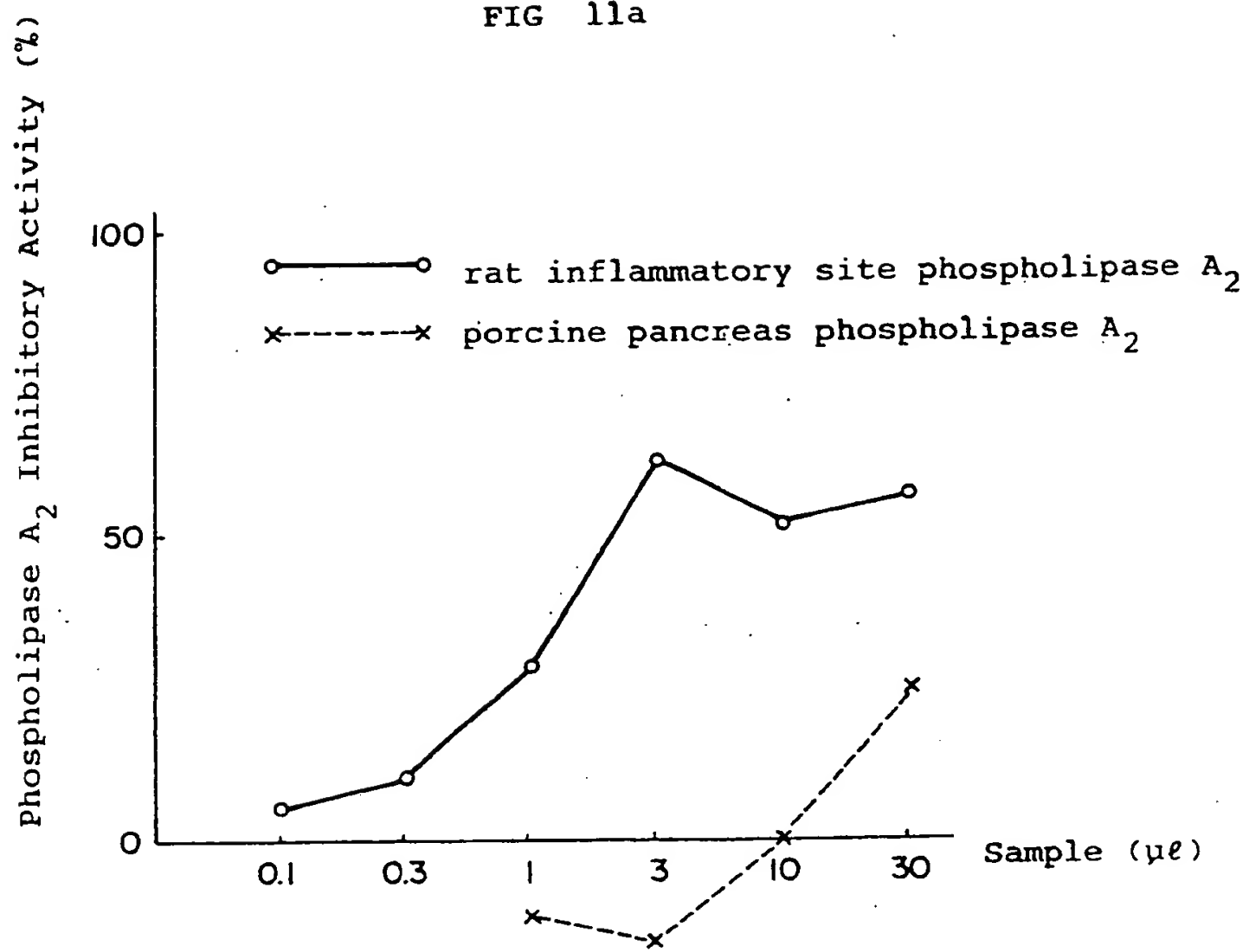


FIG 11b

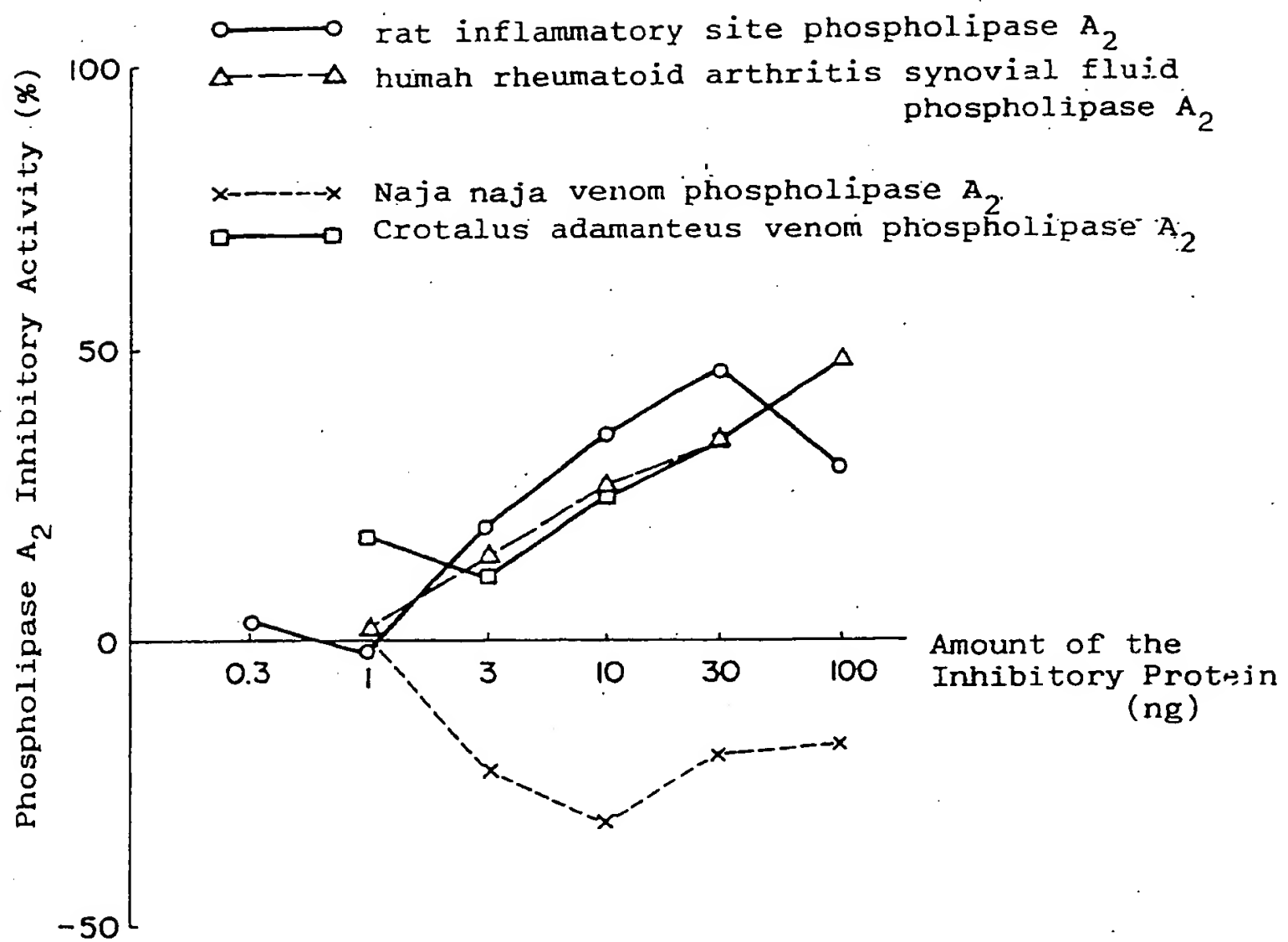
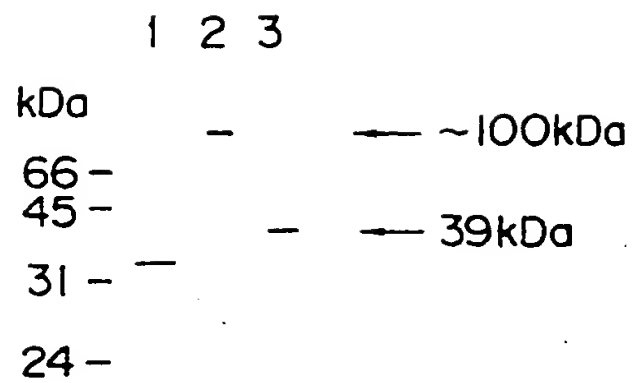


FIG 12



Lane 1 human C3a(33kDa)

2 untreated human serum (mainly ~100kDa)

3 human serum incubated at 37°C (mainly 39kDa)

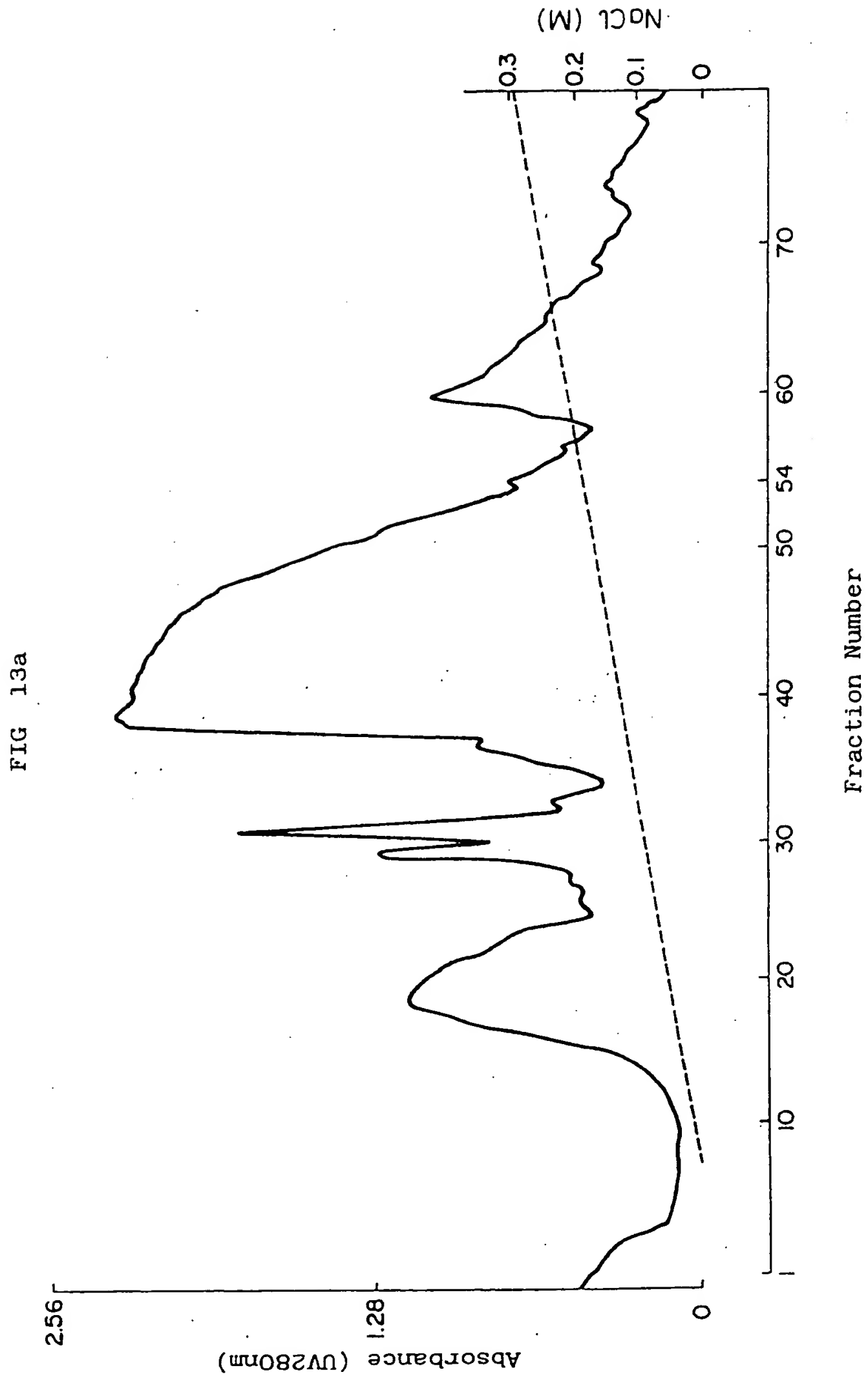


FIG 14a

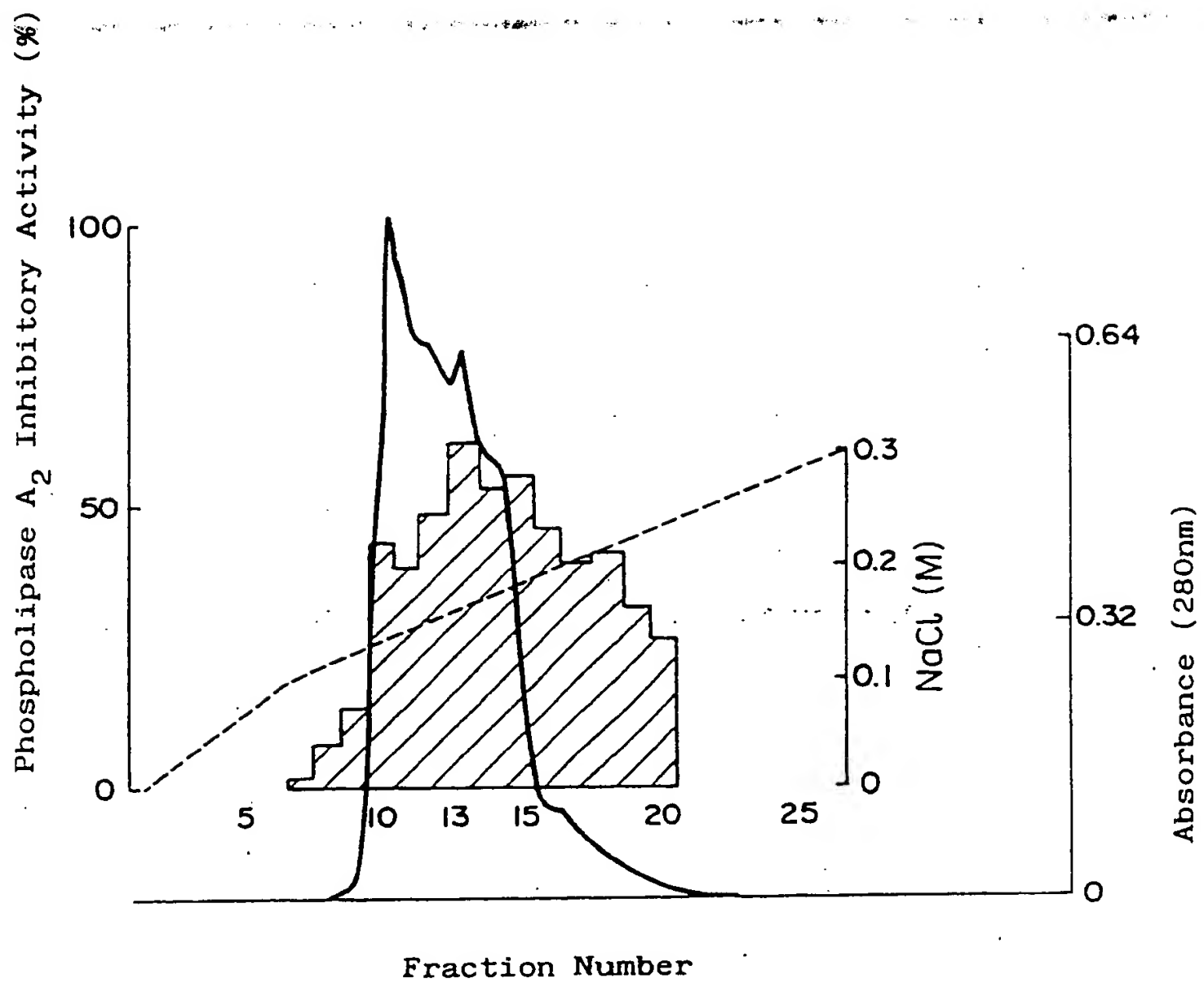


FIG 13b

Fraction Number										Marker
45	48	51	54	57	60	63	66	69		

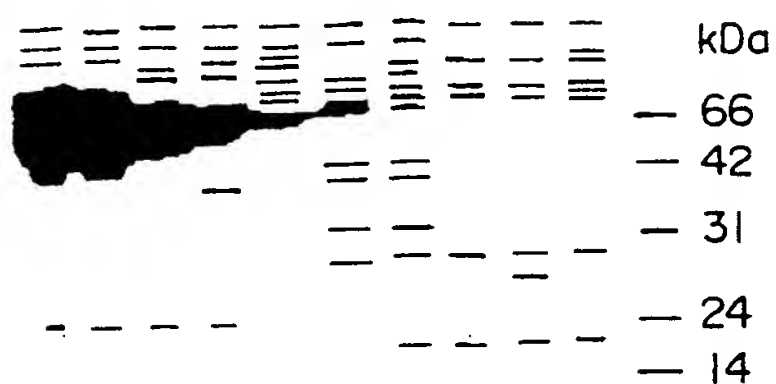


FIG 14b

Fraction Number										Marker	
9	10	11	12	13	14	15	16	17	18	19	



FIG 15a

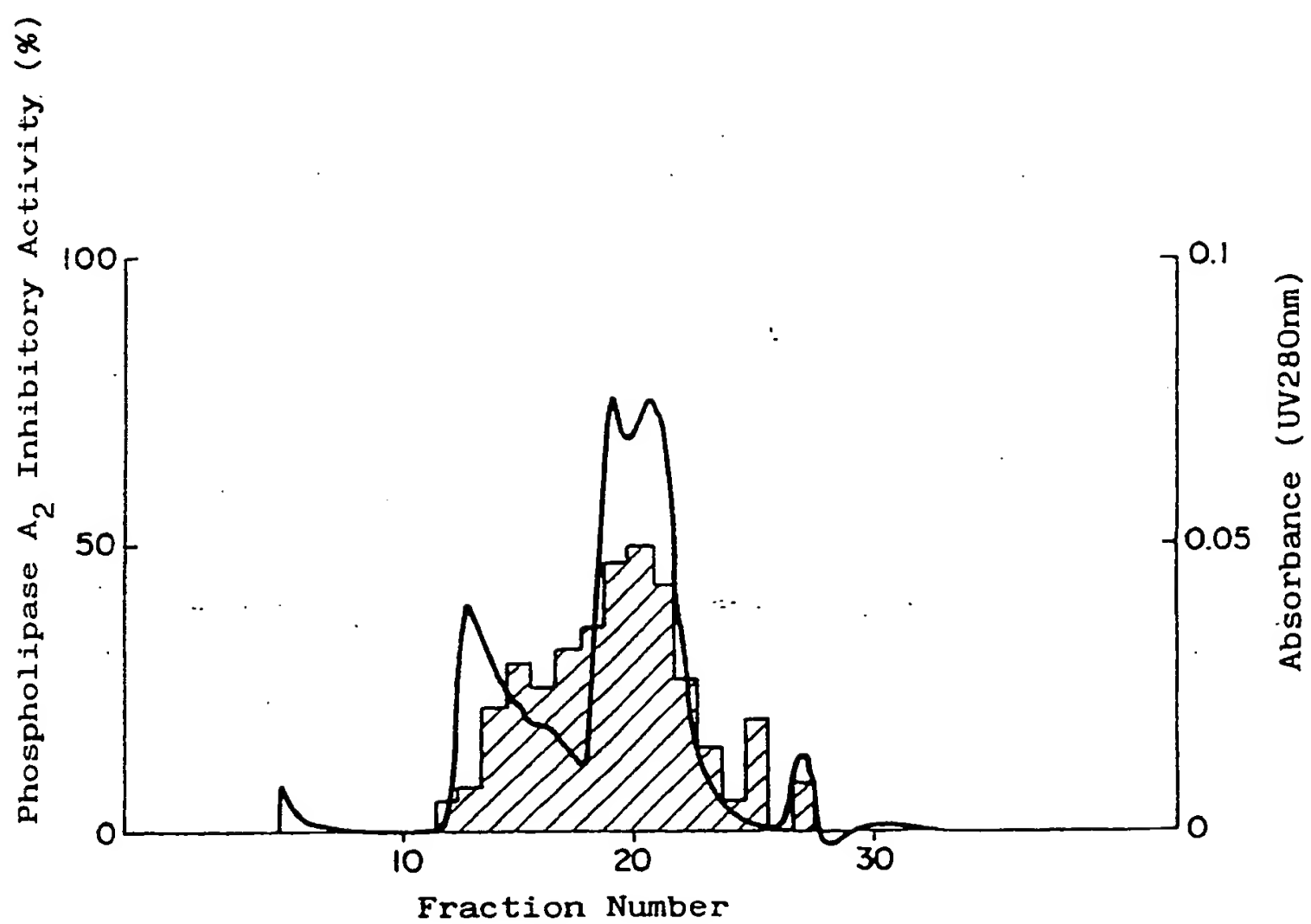


FIG 16a

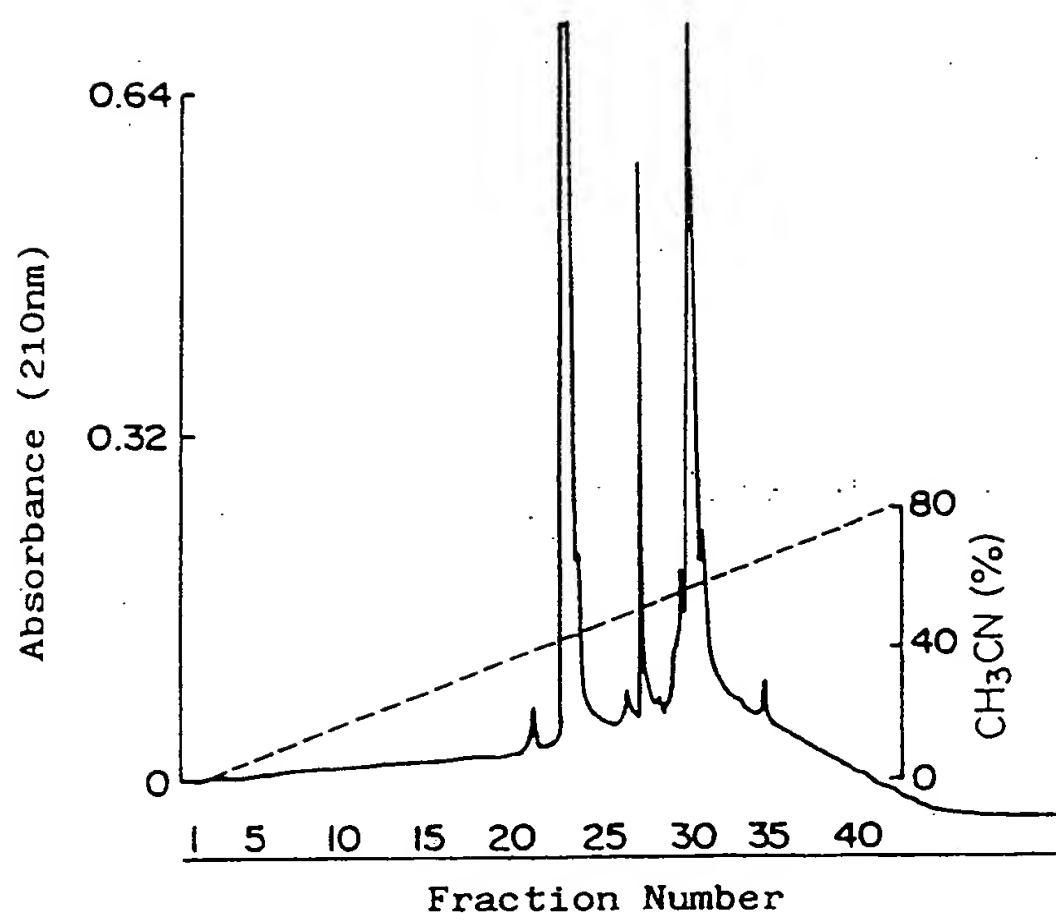


FIG 17

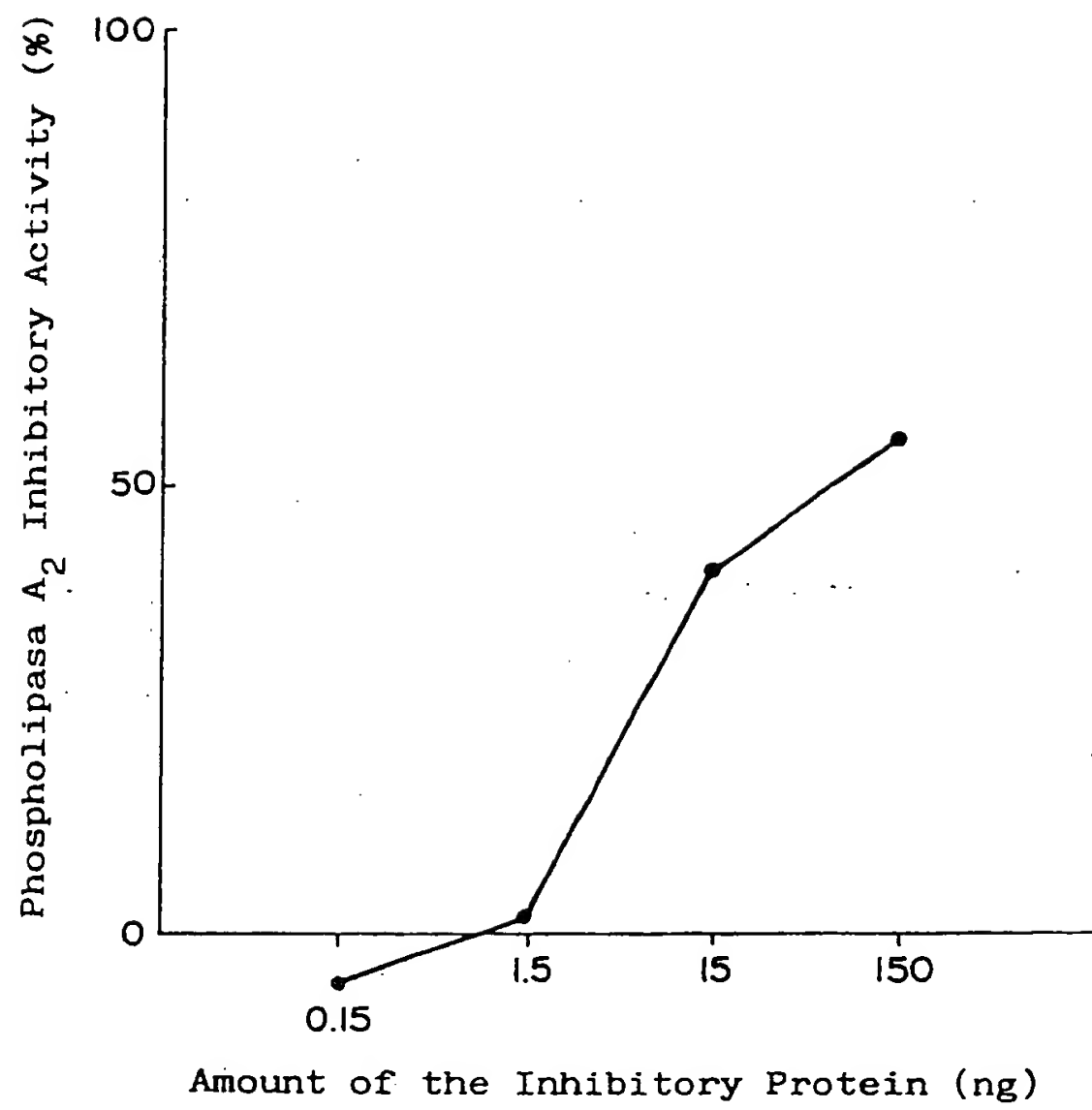


FIG 18

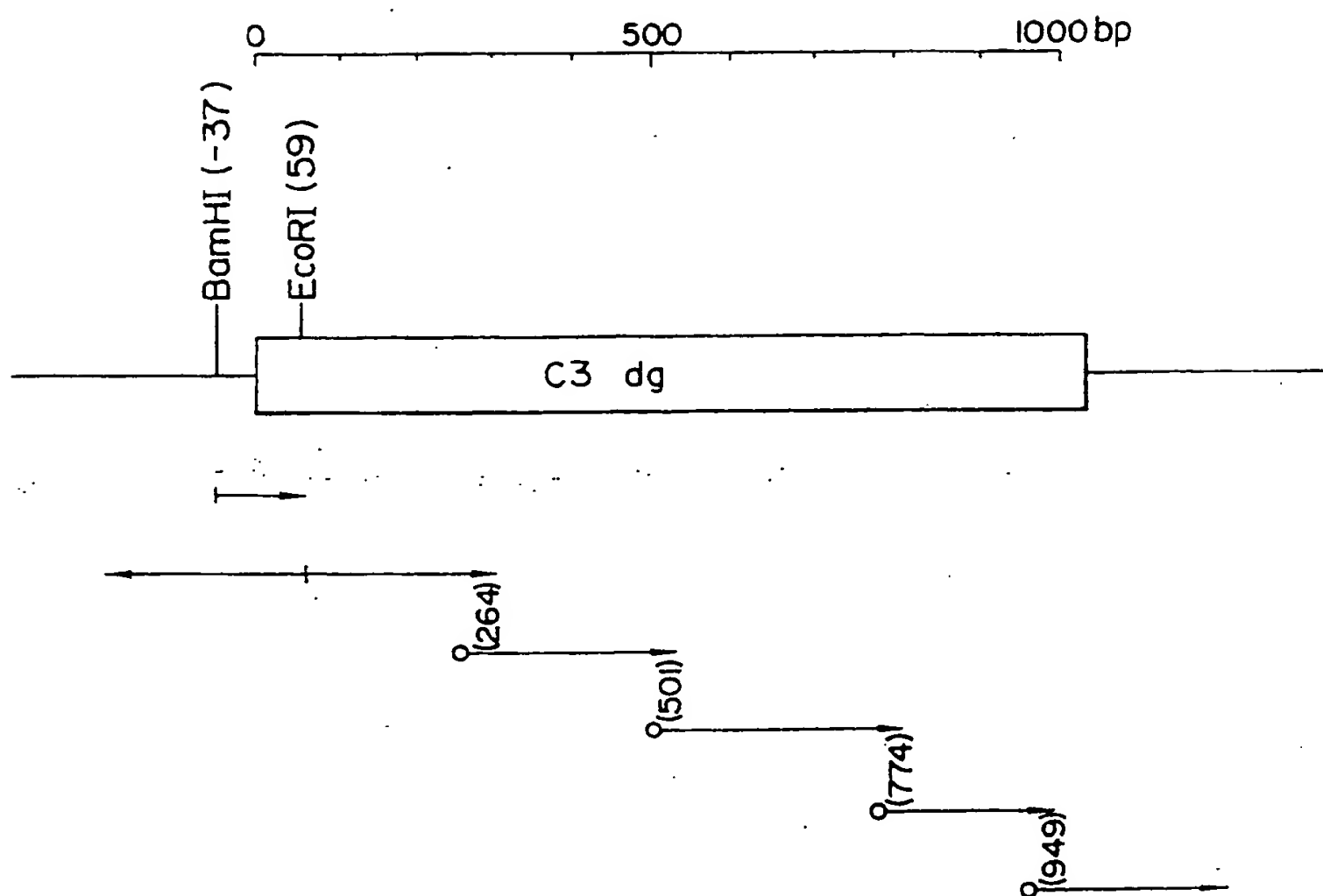
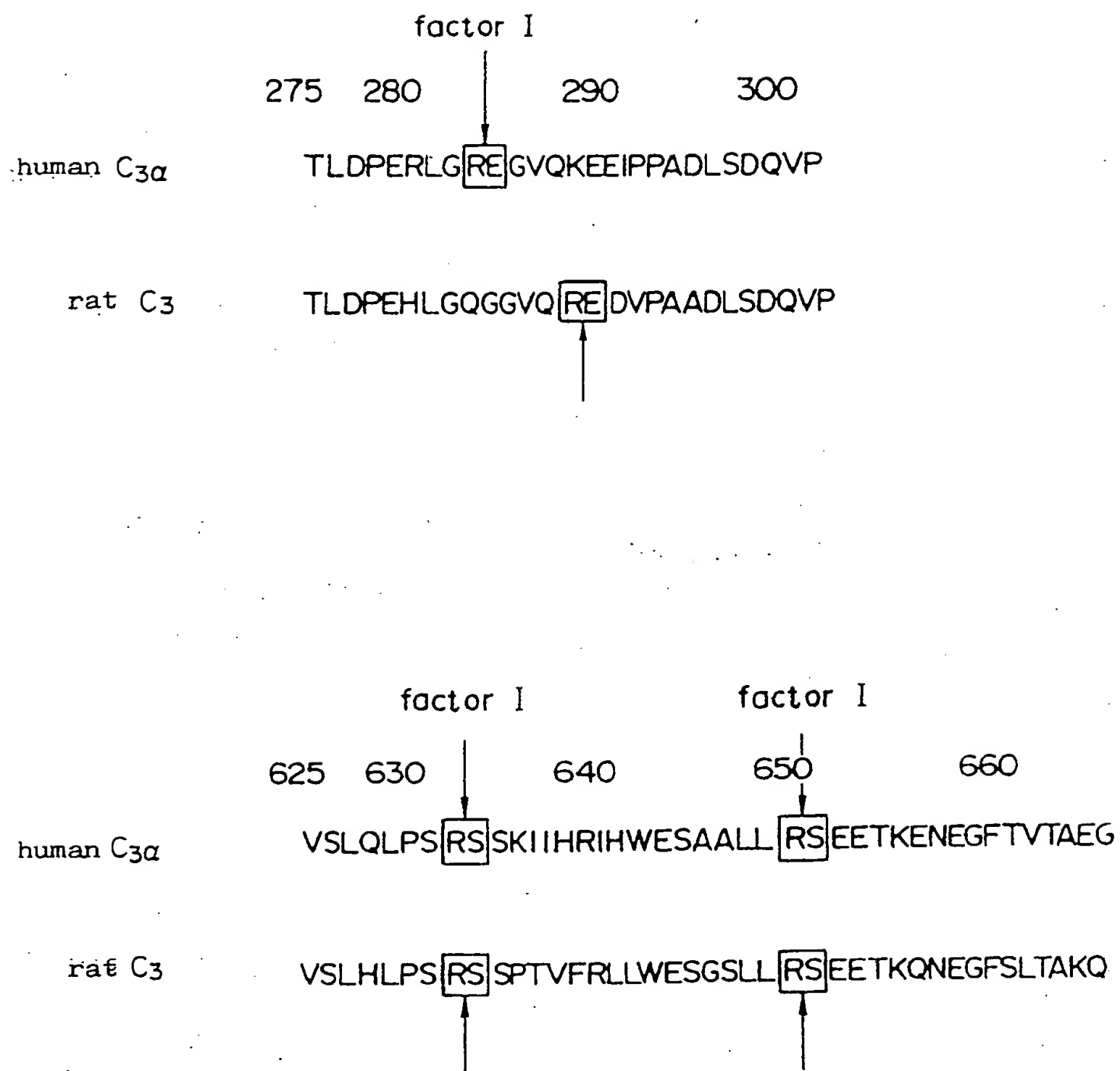


FIG 19



2

b

Western Blotting

Marker

1 2

M.W.

45000-
31000-
21500-
14400-

•

INTERNATIONAL SEARCH REPORT

International Application No PCT/JP90/00996

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int. Cl. ⁵	C07K13/00, C12P21/02, C12N15/12// C12N9/99, A61K37/02	
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
IPC	C07K13/00, 15/06, C12P21/00-21/02, C12N15/12, C12N9/99, A61K37/02	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
Biological Abstracts Data Base (BIOSIS)		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	JP, A, 63-246397 (Teijin Ltd.), 13 October 1988 (13. 10. 88) & WO, A1, 88/7452	1 - 8
X	JP, A, 63-500561 (Biotechnology Research Partneres Ltd.), 3 March 1988 (03. 03. 88) & WO, A1, 86/6100 & EP, A1, 218696	9 - 13
P	Proceedings of the National Academy of Sciences of USA, Vol.87, No.7, (April 1990), Yorimasa Suwa, et al. "Proteinaceous inhibitors of phospholipase A ₂ purified from inflammatory sites in rats" p.2395-2399	1 - 8
P	JP, A, 2-91100 (Teijin Ltd.), 30 March 1990 (30. 03. 90), (Family: none)	9 - 13
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"g" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
October 25, 1990 (25. 10. 90)	November 13, 1990 (13. 11. 90)	
International Searching Authority	Signature of Authorized Officer	
Japanese Patent Office		

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